7,8-DIHYDRONEOPTERIN INHIBITION OF OXIDISED LOW DENSITY LIPOPROTEIN-INDUCED CELLULAR DEATH

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In memory of
an inspirational father and scholar

Alexander William Stewart Baird

Splendour, simplicity, joy
Such as were seen
In one who now rests
By his mountain road.

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ABSTRACT

The death of monocyte and macrophage cells in the atherosclerotic plaque is thought to be due principally to the toxicity of oxidised LDL (oxLDL). This makes an important contribution to the development of the lipid core of advanced plaque and also to the overall stability of the plaque.

Under inflammatory conditions in which IFNγ is present, human monocytes and macrophages produce the partially reduced pteridine 7,8-dihydroneopterin (78NP). This molecule has been shown to be a very efficient free radical scavenger and to protect some cell types from damage during incubation with reactive oxygen species. The possibility of 78NP reducing loss of cell viability of monocytes and macrophages in the atherosclerotic plaque was examined in this research.

THP-1 and U937 monocytes and THP-1 macrophage-like cells were incubated with AAPH or oxidised LDL, and loss of cell viability and total reduced thiols measured. In monocytes, both AAPH and oxLDL caused concentration-dependent reduction in viability. The loss of reduced thiols was also proportional to AAPH and oxLDL concentration, except in U937 monocytes with oxLDL, where the levels dropped suddenly. Viability loss in THP-1 macrophage-like cells was found to be small and not dependent on the concentration of the AAPH or oxLDL.

The development of apoptotic features, including nuclear morphological changes, caspase enzyme activation and phosphatidylserine exposure was examined in monocytes incubated with oxLDL. Both cell types had characteristics typical of apoptosis, with the exception of low levels of caspase enzyme activity in U937 monocytes, which decreased even further during incubation with oxLDL. Evidence is presented that this may be due to the oxidation of the crucial cysteine residue on the caspase enzymes.

During incubations with AAPH, 78NP significantly reduced cell viability and thiol loss in U937 cells and decreased cell viability loss by a small amount in THP-1 monocytes when the incubations were carried out in EBSS media. 78NP had a similar effect in U937 cells with oxLDL, but no protective effect was observed in THP-1 monocytes. 78NP had no effect on viability loss with AAPH or oxLDL in THP-1 macrophage-like cells. The protective effect of 78NP may be dependent on its ability to inhibit thiol loss.

All three cell types were shown to produce 78NP after stimulation with IFNγ. However, inclusion of IFNγ did not in most cases prevent cell viability loss during exposure to oxLDL. 78NP was found in inflammatory material, but only at levels a hundred-fold less than those required to provide an antioxidant effect.

78NP is able to protect cells from viability loss generated by free radicals and the reactive oxygen species in oxLDL in some circumstances. However, since the levels *in vivo* are likely to be far lower than those required for antioxidant activity, the evidence presented does not support the hypothesis that 78NP would be able to protect monocyte and macrophage cells from cell death in the atherosclerotic plaque.

ABBREVIATIONS

3-HAA	3-Hydroxyanthranilic acid
4-HNE	4-Hydroxy-2-nonenal
6-FGF	6-Fibroblast growth factor
78NP	7,8-Dihydroneopterin
78XP	7,8-Dihydroxanthopterin
9-HODE	9-Hydroxyoctadecadienoic acid
13-HODE	13-Hydroxyoctadecadienoic acid
15-HPETE	15-Hydroperoxyeicosatetraenoic acid
AAPH	2,2'-Azobis(2-methylpropionamidine) dihydrochloride
AAS	3-Aminopropyltriethoxysilane
ABTS+	2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]
ACAT	Acyl-coenzyme A: cholesterol acyltransferase
AcLDL	Acetylated LDL
ADAF	Adult T cell leukemia-derived factor
ADP	Adenosine diphosphate
AgLDL	Aggregated LDL
AIF	Apoptosis-inducing factor
ANOVA	Analysis on variance
ANT	Adenine nucleotide translocator
AP-1	Activator protein-1
APAF-1	Apoptotic protease activating factor-1
ApoA2	Apolipoprotein A2
ApoB100	Apolipoprotein B100
ApoE	Apolipoprotein E
ASK-1	Apoptotic signal-regulating kinase-1
ATP	Adenosine triphosphate
BCA	Bincinconic acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CARD	Caspase recruitment domain

CD40	Cluster of differentiation 40
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
cGMP	Guanosine 3',5'-cyclic monophosphate
CNTP	3-Carboxylato-4-nitrothiophenolate
CSF	Colony stimulating factor
DAG	Diacylglycerol
DCFH-DA	Dichlorofluorescein diacetate
DEVD-AMC	Ac-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin)
DFF	DNA fragmentation factor
DISC	Death-inducing signalling complex
DMPO	5,5-Dimethylpyrroline-*N*-oxide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPA	3,4-Dihydroxyphenylalanine
DPI	Diphenyliodonium
DPPH	Diphenylpicrylhydrazyl
DR	Death receptor
DTNB	5 5'-Dithiobis(2-nitrobenzoic acid)
DTT	1,4-Dithiothreitol
EBSS	Earle's Balanced Salt Solution
EBV	Epstein-Barr virus
ECD	Electrochemical detector
EDTA	Ethylene diamine tetracetic acid
EPR	Electron paramagnetic resonance
ESR	Electron spin resonance
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FADD	Fas-associated protein with a death domain
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
FLICE	FADD-like ICE
FOX	Ferric-xylenol orange

GC-MS	Gas chromatography and mass spectrometry
γ-GCS	γ-glutamylcysteine synthetase
GM-CSF	Granulocyte/macrophage colony stimulating factor
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GTP	Guanosine 5'-triphosphate
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HDL	High density lipoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HETE	Hydroxyeicosatetraenoic acid
HIFBS	Heat-inactivated fetal bovine serum
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HPLC	High Performance Liquid Chromatography
Hsp	Heat shock protein
HUVECs	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis protein
ICAM	Intracellular adhesion molecule
ICE	Interleukin-1β converting enzyme
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon-γ
IκB	Inhibitor of κB
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus family kinase
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LFA-1	Leucocyte function associated antigen-1
LOOH	Lipid hydroperoxide
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LXR	Liver X receptor

MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
M-CSF	Monocyte colony stimulating factor
MDA	Malondialdehyde
MHCII	Major histiocompatibility complex II
MMP	Mitochondrial membrane permeability
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5,diphenyl tetrazolium bromide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κB
NK cell	Natural killer cell
$O_2^{\cdot-}$	Superoxide anion
$^{\cdot}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OxLDL	Oxidised LDL
OxLp(a)	Oxidised lipoprotein (a)
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAK2	p21-activated kinase 2
PARP	Poly(ADP-ribose) polymerase
PB-DOPA	Protein-bound 3,4-dihydroxyphenylalanine
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDTC	Pyrolidine dithiocarbonate
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PI	Propidium iodide
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator-activated receptor
PP2A	Protein phosphatase subfamily 2A
PS	Penicillin/streptomycin
PSSI	Phosphatidylserine synthase I
PUFA	Polyunsaturated fatty acid
REM	Relative electrophoretic mobility
RNA	Ribonucleic acid
ROOH	Protein hydroperoxide
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulphate
Smac/DIABLO	Second mitochondria-derived activator of caspases/direct IAP binding protein with low pI
SR-PSOX	Scavenger receptor that binds phosphatidylserine and oxidised lipoprotein
Stat	Signal transducer and activator of transcription
TBA	2-Thiobarbituric acid
TBARS	2-Thiobarbituric acid reactive substances
tBOOH	*Tert*-butylhydroperoxide
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor-β
Th-1	T helper 1
TNFα	Tumour necrosis factor α
TNFR1	Tumour necrosis factor receptor 1
TPA	12-O-tetradecanoylphorbol-13-acetate
TRADD	TNFα receptor-associated protein with a death domain
TRAIL	TNFα-related apoptosis inducing ligand
TUNEL	Terminal transferase-mediated dUTP-biotin nick end labelling
UTP	Uridine triphosphate
VCAM	Vascular cell adhesion molecule

VDAC	Voltage-dependent anion channel
VLDL	Very low density lipoprotein
WHHL	Watanabe heritable hyperlipidaemic
XO	Xylenol orange

INTRODUCTION

PURPOSE OF THIS STUDY

Oxidised low density lipoprotein (oxLDL) has been hypothesised to be central to the development of atherosclerosis. Tissue culture studies have shown that oxLDL can elicit many of the cellular responses that appear during the development of atherosclerotic plaque (Chisholm III and Chai, 2000). One of the key features of advanced plaque is an acellular lipid core, thought to be formed from macrophage cells that have undergone cell death (Martinet and Kockx, 2001). In tissue culture oxLDL is cytotoxic to monocytes and macrophages via a range of mechanisms (Björkerud and Björkerud, 1996).

Monocyte and macrophage cells have a number of cellular defence mechanisms which allow them to function in inflammatory environments. Some of these protective responses are activated by the T cell-synthesised cytokine interferon-γ (IFNγ) (Wachter *et al.*, 1992). 7,8-Dihydroneopterin (78NP), a pteridine synthesised from GTP, is hypothesised to be part of this response. 78NP can function as an antioxidant, scavenging a range of free radicals (Shen, 1994). It has been shown to protect some cell types from oxidative damage, and has been reported to both enhance and reduce apoptosis (Baier-Bitterlich *et al.*, 1995; Schobersberger *et al.*, 1996; Gieseg *et al.*, 2001). Pteridines may also affect cell viability through influencing the cells' signalling pathways and gene expression (Murr *et al.*, 1994).

Atherosclerotic plaques are known to be sites of inflammation (Galle *et al.*, 1999A), suggesting that the macrophages present may be synthesising 78NP. This thesis will examine the process of oxLDL-mediated cell death using two monocyte-like cell lines and one macrophage-like cell type and explore the possible inhibition of cellular death by 78NP.

MACROPHAGES AND THE GENERATION OF ANTIOXIDANTS: 7,8-DIHYDRONEOPTERIN AND INTERFERON-γ

INTERFERON-γ

The interferons are a family of cytokines, carrying messages between cells as part of the immune response. IFNα and IFNβ are type I interferons, which induce resistance to viruses (Mueller *et al.*, 1991; Johnson *et al.*, 1994; Kuby, 1997).

IFNγ is the only type II interferon. It has a dimer structure, and is much more plurifunctional than the other interferons, carrying out antiviral, anticellular, antiparasitic and immunoregulatory activities. It is produced by Th1 lymphocytes, cytotoxic T cells and NK cells activated by microorganisms or antigen, and binds to receptors on monocytes, macrophages, T or B cells (Takikawa *et al.*, 1988; Johnson *et al.*, 1994). Once bound to the cell-surface receptors, it activates the Janus tyrosine kinases, which phosphorylate Stat factors 113, 91 and 84. These can then form a complex and activate various genes (Johnson *et al.*, 1994).

IFNγ enhances the Th1 type immune response by increasing inflammation via macrophages, which are then better able to ingest and kill invading, infected or cancerous cells (Kuby, 1997; Widner *et al.*, 2000). IFNγ primes the respiratory burst and induces expression of the MHC II antigen, MHCI, FcR, IL-1, IL-15, TNFα, platelet activation factor, adhesion molecules, proteases and iNOS, and enhances the production of complement factors B, C2 and C1-inhibitor (Faltynek *et al.*, 1988; Billiau and Dijkmans, 1990; Kuby, 1997; Musso *et al.*, 1999).

Since it is part of the cytokine network, IFNγ's production is modulated by many other endogenous factors, such as interleukins, other interferons and lymphotoxins (Billiau and Dijkmans, 1990). Anti-inflammatory cytokines diminish its effects. Histamine, Fe^{2+} ions, Fe^{3+} ions, transferrin and metalloporphyrins containing iron or zinc reduce IFNγ effects in THP-1 monocytes (Weiss *et al.*, 1992; Weiss *et al.*, 1993B; Gruber *et al.*, 2000).

To counter some of the inflammatory effects, IFNγ also increases production of molecules which can act as antioxidants. It induces the degradation of heme to bilirubin (Christen *et al.*, 1994), transiently induces ceruloplasmin secretion in U937 cells and

enhances it in peripheral blood monocytes and THP-1 monocytes, which produce higher constitutive levels (Mazumder *et al.*, 1997). Pteridines are also produced (Huber *et al.*, 1984), and metabolism of tryptophan is increased (Wachter *et al.*, 1992).

PTERIDINES: BACKGROUND

Pteridines were first described in 'Nature' in 1889. They are pyrazino-pyrimidine compounds, ubiquitous and essential for many cell functions in plants, bacteria, non-mammals and mammals (Woell *et al.*, 1993; Walter *et al.*, 2001). Their synthesis is stimulated by interferons, in particular IFNγ. IFNα, IFNβ, TNFα, TNFβ, IL-1, IL-2, IL-6, GM-CSF, LPS, lectins, PMA, zymosan and Ca^{2+} ionophore A23471 can also increase the production, but not by as great a degree as IFNγ. Some of these cytokines are able to act in synergy with IFNγ and enhance its effects (Troppmair *et al.*, 1988; Meyer *et al.*, 1992; Werner-Felmayer *et al.*, 1993).

Pteridines can be either conjugated, with a relatively complex side chain, such as folic acid and riboflavin, or unconjugated, with a less complex side chain at position 6, including biopterin and neopterin (Wachter *et al.*, 1989). The unconjugated pteridines can be grouped into three classes by oxidation state: fully reduced tetrahydrobiopterins, partially reduced dihydropterins and aromatic pterins such as neopterin and biopterin (Oettl and Reibnegger, 2002).

The biosynthetic pathway begins with the cleavage of the purine GTP by GTP cyclohydrolase I to form 7,8-dihydroneopterin triphosphate (Figure 1) (Wachter *et al.*, 1992). 7,8-dihydroneopterin triphosphate is a precursor to folic acid and riboflavin in microorganisms. Higher animals and mammals synthesise pteridines instead (Wachter *et al.*, 1989).

GTP cyclohydrolase I is a *d5* symmetric toroid-shaped decamer, with ten active sites at the interface of three adjacent subunits, and an essential Zn^{2+} ion bound to conserved cysteine and histidine residues at each active site. The reaction is complex, with ten proposed steps. The sequence begins with the opening of the imidazole ring of GTP, and continues with a carbohydrate rearrangement and ring closure (Schramek *et al.*, 2002).

GTP cyclohydrolase I is the only enzyme in the pathway modulated by IFNγ and other cytokines, without which its levels are low and rate-limiting. The rate-limiting part of the reaction is associated with the rearrangement of the carbohydrate side chain. IFNγ also increases the levels of GTP itself (Kojima *et al.*, 1992). Once GTP cyclohydrolase I is activated, the levels of the other enzymes in the pathway determine the identity of the final products (Werner *et al.*, 1990). This will vary between cell types (Wachter *et al.*, 1992).

If 6-pyruvoyl tetrahydropterin synthetase, which metabolises 7,8-dihydroneopterin triphosphate, is active at high levels, the main product will be 5,6,7,8-tetrahydrobiopterin. 6-Pyruvoyl tetrahydropterin synthetase generates 6-pyruvoyl-tetrahydropterin, and sepiapterin reductase reduces it to 5,6,7,8-tetrahydrobiopterin, which can be metabolised to biopterin and 7,8-dihydrobiopterin (Walter *et al.*, 2001).

If 6-pyruvoyl tetrahydropterin synthetase levels are low, the 7,8-dihydroneopterin triphosphate is likely to be metabolised by less specific enzymes (Wachter *et al.*, 1992). Aldoses can convert 7,8-dihydroneopterin triphosphate to 7,8-dihydrofolic acid (Spoettl *et al.*, 1999/2000). Phosphatase activity results in 7,8-dihydroneopterin (78NP) (Wachter *et al.*, 1992). Humans and other primates are the only species in whose body fluids 78NP has been detected (Wachter *et al.*, 1989).

78NP is oxidised to two different products, depending on the oxidant. HOCl or acidic iodine solution oxidises 78NP to neopterin by the loss of hydrogen at C7 and N8 (Gieseg *et al.*, 2000B; Widner *et al.*, 2000). The other product is 7,8-dihydroxanthopterin, which comes about through loss of the trihydroxypropyl side chain at position 6. This may occur by a retro-aldol reaction, which could be initiated by the abstraction of a hydrogen atom from the central hydroxyl group on the side chain (Murr *et al.*, 1994; Widner *et al.*, 2000; Duggan *et al.*, 2002). It is formed by peroxyl radicals and hydrogen peroxide (Gieseg *et al.*, 2000B).

Neopterin and 7,8-dihydroneopterin are found in reasonably constant proportions in human body fluids, with a ratio of neopterin: total neopterin (where the 78NP has been oxidised to neopterin) of 1:3 in urine and 1:2 in serum from venous blood samples. There are higher 78NP levels in blood from arteries and in cerebrospinal fluid (Werner-Felmayer *et al.*, 1990). Pterins are cleared only by the kidneys (Widner *et al.*, 1999), which is why the overall levels in urine are higher than those in serum (Mueller *et al.*, 1991).

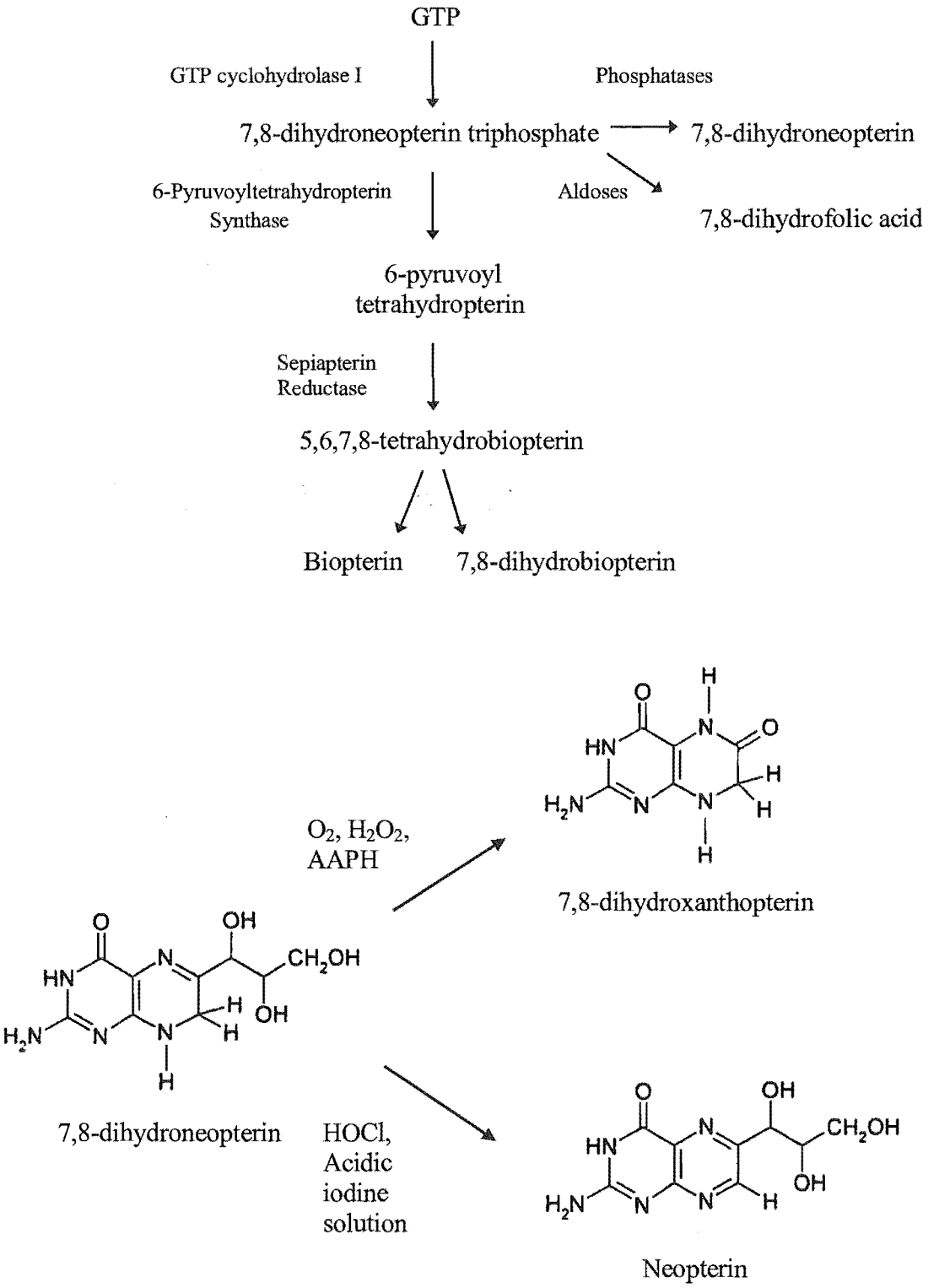


Figure 1: The biosynthesis and metabolism of 7,8-dihydroneopterin. Adapted from Spöttl *et al.*, 1999/2000 and Widner *et al.*, 2000.

The production of 78NP and neopterin correlates closely with IFNγ serum concentration and activation of cell-mediated (Th1) immunity. As neopterin is very fluorescent, the determination of its levels in body fluids is a valuable diagnostic tool in conditions associated with increased activation of the cellular immune system (Hoffman *et al.*, 1996).

Examples in which an increase is found in serum include autoimmune diseases, such as rheumatoid arthritis, ulcerative colitis, Crohn's disease, autoimmune thyroiditis (Wachter *et al.*, 1989), rejection of transplanted organs including liver, renal, cardiac, pancreatic and bone marrow transplants (Mueller *et al.*, 1991) and viral, bacterial and protozoal infections, (Huber *et al.*, 1984; Werner-Felmayer *et al.*, 1990; Strohmaier *et al.*, 1996), including malaria (*Plasmodium falciparum*) (Facer, 1995), hepatitis (Woell *et al.*, 1993) and HIV (Fuchs *et al.*, 1989; Griffin *et al.*, 1991; Moriuchi *et al.*, 2000). Increases are also found in diabetes mellitus type I, inflammatory diseases such as sarcoidosis, celiac disease, multiple sclerosis and aseptic meningoencephalitis, cancers including gynecological, genitourinary tract, lung, gastrointestinal, pancreatic and hematological neoplasias (Wachter *et al.*, 1989), alcoholic liver disease, burn patients (Facer, 1995), pregnancy (Widner *et al.*, 1999), in breastfeeding women with silicone breast implants (Levine *et al.*, 1996) and long-term hemodialysis patients with immune activation due to the interaction of the blood with the membrane (Fuchs *et al.*, 1988). Increased levels are measured in the cerebrospinal fluids of neurodegenerative patients with Alzheimers, Huntington's or cerebral infection, such as Lyme neuroborreliosis (Sattler *et al.*, 1999; Spoettl *et al.*, 1999/2000).

The rise in neopterin levels does not necessarily occur in every patient. In cancer, the frequency of elevated neopterin varies from 18% of patients with breast cancer to 100% in some hematological neoplasias (Murr *et al.*, 1999). Some studies also show increases in neopterin which are very small, although apparently still statistically significant. It is not clear whether this rise would be physiologically important. For example, in a study of Hodgkin's disease and non-Hodgkin's lymphomas, the highest increase found was 1.1-fold (Denz *et al.*, 1992), yet the increase was still said to be predictive of the cancer.

Higher levels of neopterin are generally related to poor prognosis (Facer, 1995), correlating with more rapid progression and earlier death (Baier-Bitterlich *et al.*, 1997). A decline is found in the remission of cancers (Murr *et al.*, 1999).

POSSIBLE ROLES OF PTERIDINES *IN VIVO*

Some pteridines have been assigned biological roles, usually as enzyme cofactors. Tetrahydrofolate is a cofactor in thymine synthesis and in the conversion of homocysteine to methionine (Wachter *et al.*, 1989; Widner *et al.*, 2002). Pterin is a component in the molybdenum cofactor of nitrate reductase, sulphite oxidase and xanthine oxidase (Wachter *et al.*, 1989; Spoettl *et al.*, 1999/2000). 5,6,7,8-Tetrahydrobiopterin is a cofactor of aromatic amino acid monooxygenases, etherlipidoxidase (Werner-Felmayer *et al.*, 1990; Mueller *et al.*, 1991; Woell *et al.*, 1993) and nitric oxide synthase isoforms (Werner-Felmayer *et al.*, 1993; Walter *et al.*, 2001), as well as having a role in cell differentiation and proliferation (Walter *et al.*, 2001).

The role of 78NP and neopterin is much less certain. Several ideas have been put forward and tested, but it is not yet proven whether any of them actually occur *in vivo*. The most often explored possible roles for 78NP and neopterin concern their pro- or anti-inflammatory effects. This type of function would make sense, given that they are generated by monocytes and macrophages under inflammatory conditions. They may be able to modulate those conditions, protecting the macrophage itself, or enhancing its cytotoxicity. These effects are most often due to their pro- or antioxidant activities.

For example, 78NP could act as an extra- or intracellular antioxidant to protect the cell against its own oxidants. A similar level of IFNγ is required for maximum 78NP release as well as maximum reactive oxygen species production, and the timecourse to 78NP and hydrogen peroxide secretion is similar. The protection would also extend to other oxidants that are produced in inflammation, such as those of neutrophils. Inflammatory sites have high concentrations of superoxide and HOCl, as well as hydrogen peroxide (Nathan, 1986). 78NP may be one of the many safeguards in place to ensure the process does not get out of hand. This is the possibility investigated in the research of this thesis.

78NP might also improve macrophage immune function, as well as protecting the cell. The antioxidants glutathione, N-acetylcysteine, vitamin E and vitamin C stimulated phagocytosis, increased superoxide production and improved migration and chemotaxis *in vitro*. Vitamins E and C increased levels of adherence (Tillinghast and Newell, 1987; Del Rio *et al.*, 1998). Also, the ratio of neopterin and 78NP can influence the balance between superoxide and NO. This is crucial to the development of peroxynitrite. At the right pH, neopterin will increase its toxicity by enhancing the nitration of tyrosine (Shen, 1994).

It has been proposed (but not tested) that these pterins may function to increase the macrophage's arsenal against invaders by inhibiting some of the foreign enzymes. Neopterin is an intermediate in the folic acid pathway, which is part of microorganisms', but not animals', metabolism. Neopterin might therefore inhibit folate synthesis. At least two enzymes in the pathway use dihydro substrates, so neopterin, the oxidised form, may inhibit them, or at least dilute the pool of available substrate (Nathan, 1986). 78NP may have a similar function, as it seems able to competitively inhibit methyl group metabolism enzymes, such as dihydrofolate reductase and dihydropteridine reductase, which recycle the pterin cofactors used for forming tetrahydroderivatives. 78NP must first be tautomerised to the 7,8(6H)-dihydro derivative by Fe^{3+} (Fuchs *et al.*, 1990).

Many studies have been carried out showing that pterins can interfere with a redox balance, sometimes within cells and sometimes in purely chemical systems. Their exact effect depends on their structure, concentration and the system they are in. The same amount of a pterin can have the opposite effect in a different system.

PTERIDINES AS PRO- AND ANTIOXIDANTS, OR REDOX MODULATORS

In general, aromatic pterins such as neopterin act in a pro-oxidant, pro-inflammatory fashion, and partially reduced pterins such as 78NP and fully reduced pterins such as 5,6,7,8-tetrahydrobiopterin are antioxidants and anti-inflammatory, except at very high concentrations or in cell types which do not produce them themselves.

Neopterin's prooxidant effect was shown when it enhanced chloramine-T and hydrogen peroxide-mediated chemiluminescence in a luminol assay at concentrations between 2.0nM and 200μM (Murr *et al.*, 1994; Baier-Bitterlich *et al.*, 1995). In a solution of

tyrosine, nitrite and hydrogen peroxide, 200μM neopterin enhanced 3-nitrotyrosine formation (Widner *et al.*, 1998). Neopterin also sensitised DNA to photodamage through the formation of pterin radicals (Oettl and Reibnegger, 2002).

Any apparent antioxidant activity of neopterin has so far been found to be due to inhibition of the enzyme generating the oxidative stress, rather than a chain-breaking or scavenging activity (Oettl and Reibnegger, 2002). Neopterin inhibited superoxide generation by the NADPH oxidase enzyme, by competing with NADPH (Kojima *et al.*, 1993) and by non-competitive, reversible inhibition of the xanthine oxidase enzyme (Oettl *et al.*, 1997; Watanabe *et al.*, 1997; Oettl and Reibnegger, 1999). The inhibition of xanthine oxidase may be related to the fact that pterin forms part of the enzyme's cofactor (Wachter *et al.*, 1989; Spoettl *et al.*, 1999/2000). Concentrations *in vivo* are not likely to be high enough to inhibit these enzymes (Kojima *et al.*, 1993).

78NP, a partially reduced pterin, has mainly scavenger and protectant properties. It has been shown to scavenge superoxide in xanthine/xanthine oxidase or NADPH cytochrome P450 reductase systems, with an IC_{50} of 3.85μM (Shen, 1994; Oettl *et al.*, 1997). The IC_{50} was 0.16μM for 78NP in a glucose/glucose oxidase system generating hydrogen peroxide and 78NP also scavenged hydrogen peroxide-mediated chemiluminescence (Murr *et al.*, 1994; Shen, 1994; Baier-Bitterlich *et al.*, 1995). Hydroxyl radicals generated using a chloramine system were quenched by 50% by 78NP (Oettl and Reibnegger, 2002). EPR spectroscopy showed 78NP was as effective at scavenging DPPH as Trolox, and more effective than Trolox with ABTS+ (Oettl *et al.*, 2000A). Using AAPH to generate peroxyl radicals, 78NP reduced the radical level to 11%. The rate constant for the 78NP reaction was 10^7 M^{-1} s^{-1}, which is close to α-tocopherol (Oettl *et al.*, 1997). 78NP could also scavenge peroxynitrite as measured by EPR spectroscopy (Oettl *et al.*, 2000A).

78NP inhibited peroxyl and hydroxyl radical-mediated protein hydroperoxide formation on bovine serum albumin completely at 200μM. It could also degrade preformed protein hydroperoxides on BSA (Duggan *et al.*, 2002). 78NP at 200μM inhibited 3-nitrotyrosine formation in a solution of tyrosine, nitrite and hydrogen peroxide at any pH (Widner *et al.*, 1998).

78NP's antioxidant capacities have also been tested in LDL oxidation. Between 1μM and 10μM 78NP could prevent the oxidation of linoleate and LDL with AAPH and Cu^{2+}, increasing the lag phase of lipid peroxidation, and diminishing the consumption of vitamin E. 78NP appeared to be acting as a chain-breaking antioxidant, effectively competing with vitamin E for the lipid peroxyl radical. It was suggested that it was unlikely the 78NP was acting to directly scavenge the peroxyl radicals, as it had a similar efficiency of protection with Cu^{2+} (Gieseg *et al.*, 1995).

The fully reduced 5,6,7,8-tetrahydrobiopterin has even more impressive antioxidant properties than 78NP, although it is not known if it has an antioxidant function *in vivo*. It inhibits luminol-dependent chemiluminescence in all enzyme or chemical systems examined. It was able to scavenge superoxide and hydroxyl radicals, DPPH and hydrogen peroxide more efficiently than established antioxidants like ascorbate and glutathione (Kojima *et al.*, 1993; Shen, 1994; Kurobane *et al.*, 1995).

5,6,7,8-Tetrahydrobiopterin is very readily oxidised in air (autooxidation) (Arai *et al.*, 1998) and is a strong reducing agent, reducing ferric to ferrous ions, which gives it a pro-oxidant ability to enhance Fenton reactions. Mass spectroscopy has shown that 5,6,7,8-tetrahydrobiopterin and iron ions are able to form complexes (Oettl and Reibnegger, 2002).

78NP is less prone to autooxidation than the fully reduced tetrahydrobiopterins. However, it is still sensitive to oxidation, especially with transition metals. Hydroxyl radical formation was illustrated by the hydroxylation of salicyclic acid in solutions of 500μM 78NP without another radical generator, as ferric ions were reduced to ferrous. Other dihydropterins yielded different degrees of hydroxylation, dependent on their redox potential and structure. Superoxide was thought to be formed from dissolved oxygen by autooxidation of trace metals, with subsequent production of hydroxyl radicals through Fenton reactions. The addition of chelated ferric ions increased the hydroxyl radical yield ten-fold, possibly due to iron binding by the 78NP. The side chain at position 6 was important here (Oettl *et al.*, 2000B).

Hydroxyl radicals were also shown to be formed dose-dependently in HEPES with 200μM-5mM 78NP by ESR spectroscopy, using the spin trap nitrone compound DMPO. A

reduced signal was found in chelexed HEPES, supporting the theory advanced above (Wirleitner *et al.*, 2001). These same properties are responsible for the fact that 5mM 78NP has been shown to enhance chemiluminescence (Oettl and Reibnegger, 2002).

78NP can also reduce ferric to ferrous ions in heme proteins, both in hemoglobin and myoglobin, at concentrations as low as 40μM. This allows molecular oxygen to bind and 78NP then supports the cleavage of the porphyrin ring, to produce carbon monoxide and non-heme iron. The reduction of iron is a potent electron-generating system (Horejsi *et al.*, 2002). 78NP may have a role in reducing the Fe^{3+} of transferrin, elevated in IFNγ-induced anaemia, which is necessary for iron binding (Fuchs *et al.*, 1991; Denz *et al.*, 1992).

78NP may also have its own ion binding capacity, although this has proved difficult to demonstrate so far (Oettl, personal communication, 2002). The previously described work with LDL oxidation suggests it may not bind Cu^{2+}, since it had no effect on the propagation rate of the oxidation with Cu^{2+} (Gieseg *et al.*, 1995). Other pteridines, including 5,6,7,8-tetrahydrobiopterin and the pteridine carboxaldehyde thiosemicarbazones, are able to chelate iron. The pteridine carboxaldehyde thiosemicarbazones inhibit enzymes in this way. Amino and hydroxyl groups were found to be important for this property (Hochmann *et al.*, 1973).

78NP's dual role in radical scavenging and formation under different conditions was well illustrated in an experiment in which, in a phosphate buffer with low iron concentrations, 78NP enhanced superoxide formation over a time period of hours. However, in organic buffer, with a high iron concentration, a reduction in superoxide production was found after ten minutes (Oettl and Reibnegger, 2002).

To summarise, 78NP and neopterin are both redox active compounds which can act as pro- or antioxidants depending on the circumstances, although 78NP is usually an antioxidant and neopterin usually a prooxidant. They are likely to be able to modulate the redox balance in cells and in the oxidative microenvironment of the inflammatory site.

EFFECTS ON CELL VIABILITY AND APOPTOSIS

The effect of the pterins on cell viability parallels their effect on radicals and reactive oxygen species. Neopterin may enhance or not affect cell viability loss, and 78NP reduces viability loss, except at high concentrations. This implies the existence of a relationship between cell death and changes in the cellular redox balance.

Neopterin seems able to enhance apoptosis, but not cause it itself. Neopterin at 200μM enhanced the toxicity of chloramine-T at pH 7.5 towards *Escherichia coli* and *Staphylococcus aureus* (Weiss *et al.*, 1993A; Murr *et al.*, 1994), increased cell damage on B-16 melanoma cells by UV-A irradiation (Murr *et al.*, 1999) and, at 1mM, enhanced apoptosis in the rat alveolar epithelial L2 cell line induced by IFNγ and TNFα through the production of NO (Schobersberger *et al.*, 1996). These cells do not produce 78NP or neopterin themselves. This is likely to explain the discrepancy with neopterin up to 1mM slightly decreasing TNFα-induced apoptosis in U937 monocyte cells (Baier-Bitterlich *et al.*, 1995).

78NP can also inhibit apoptosis in different cell types. In U937 cells undergoing TNFα-induced apoptosis, 78NP was a potent inhibitor up to a concentration of 300μM (Baier-Bitterlich *et al.*, 1995). 78NP up to 1mM inhibited apoptosis in Jurkat T cells by anti-Fas antibodies (Wirleitner *et al.*, 1998).

High concentrations of 78NP cause or enhance apoptosis, much as they augmented oxidative stress. In the rat alveolar epithelial L2 cell line, 78NP at concentrations up to 1mM increased apoptosis induced by IFNγ and TNFα through the production of NO (Schobersberger *et al.*, 1996). 5mM 78NP superinduced apoptosis of U937 cells with TNFα, by boosting radical production (Baier-Bitterlich *et al.*, 1995) and also caused apoptosis in a rat pheochromocytoma cell line (Enzinger *et al.*, 2001), PC12 cells (Enzinger *et al.*, 2002), human neuronal NT2/HNT cells (Spoettl *et al.*, 1999/2000) and Jurkat T cells, which could be abrogated by antioxidants (Baier-Bitterlich *et al.*, 1996A; Wirleitner *et al.*, 1998; Wirleitner *et al.*, 2001).

78NP can protect cells from viability loss due to oxidative damage. It shielded red blood cells from haemolysis initiated by AAPH and HOCl, and U937 cells from HOCl and Fe-mediated damage. Effects mediated by hydrogen peroxide could only be partially reduced (Gieseg *et al.*, 2000B; Gieseg *et al.*, 2001).

In line with its strong antioxidant capability in chemical systems, 5,6,7,8-tetrahydrobiopterin can also protect cells and whole animals from oxidative damage. It protected endothelial cells from cumene hydroperoxide-induced cell viability loss, bringing them back up to control levels by 1mM (Kurobane *et al.*, 1995). It was able to shield linoleic acid from air oxidation (Kojima *et al.*, 1993). 5,6,7,8-Tetrahydrobiopterin had a protective effect in free radical-induced injury in developing paw edema, gastric ischemia reperfusion injury, adriamycin cardiotoxicity and carbon tetrachloride heptatoxicity in mice (Arai *et al.*, 1998).

EFFECTS ON GENE EXPRESSION AND CELL SIGNALLING

Part of the pterins' possible influence on inflammation may occur through alteration of the cells' patterns of gene expression, either directly or more indirectly via cell signalling. Neopterin inhibited hypoxia-induced erythropoietin gene expression and formation in HepG2 cells (Murr *et al.*, 1999) and induced proto-oncogene *c-fos* expression (Schobersberger *et al.*, 1996) via effects on cytokines and second messengers, mostly mediated by reactive oxygen species.

Both neopterin and 78NP are able to enhance the effects of second messenger cGMP. Together, they induced *c-fos* in NIH 3T3 fibroblasts (Murr *et al.*, 1999). Another study also looking at fibroblasts, with a transfected *c-fos* construct, showed 78NP and cGMP causing significant transcription activation of the construct. Antioxidant activity may be important, as BHT and BHA could also have this effect (Ueberall *et al.*, 1994).

Neopterin activated iNOS in rat vascular smooth muscle cells at 10μM (Hoffmann *et al.*, 1998). In human vascular smooth muscle cells, neopterin had an additive effect on iNOS expression if stimulated with LPS. 78NP had no effect, and neither of them changed the induction by IFNγ (Schobersberger *et al.*, 1995). Conversely, another study showed that if

IFNγ or TNFα mediated the iNOS stimulation, neopterin would decrease the activation (Hoffmann *et al.*, 1998). Neopterin cannot stimulate iNOS in the rat alveolar epithelial cell line L2 (Schobersberger *et al.*, 1996).

The iNOS activation occurs through a modulation of the redox-modulated signalling pathways which induce it, so the pterin's pro- and antioxidant properties are important. Oxidative stress activates NFκB, for which the iNOS gene has two putative binding sites. Neopterin increased nuclear uptake of NFκB through inducing TNFα, which in turn induced free radical production and caused the release of NFκB from IκB (Hoffman *et al.*, 1996). Neopterin could also activate NFκB in Jurkat cells (Barak and Gruener, 1991; Schobersberger *et al.*, 1996).

78NP at 200μM was able to induce an increase in IFNγ secretion by Jurkat T cells, in what would be a self-amplifying feedback loop (Baier-Bitterlich *et al.*, 1996B). But if hydrogen peroxide, which also induces it, was present, 200μM 78NP decreased secretion, whereas 5mM superinduced it, in another example of its biphasic effects (Baier-Bitterlich *et al.*, 1996B). Extra IFNγ might result in proinflammatory events.

Pterins could also affect signalling by influencing another second messenger: Ca^{2+}. When pterins were added to THP-1 cells, a rapid transient increase in Ca^{2+}, lasting for around one minute, was found. 78NP was the most efficient pterin, requiring only 10nM for a significant change. 100nM 5,6,7,8-tetrahydrobiopterin and 1μM neopterin had the same effect. The increase was blocked by lanthanum ions, showing the involvement of an inducible calcium channel activated by the pteridines (Woell *et al.*, 1993). In another investigation, using alveolar epithelial cells, neopterin was found to abolish an ATP-stimulated intracellular Ca^{2+} release (Hoffmann *et al.*, 2002).

CELL DEATH: APOPTOSIS AND NECROSIS

Cell death is commonly divided into two categories: apoptosis and necrosis. In a certain situation, a cell's loss of viability is assigned to one or the other, depending on the appearance of certain features.

Necrosis, sometimes called oncosis, was the earliest form of cell death described. It is usually associated with a sudden or high level of damage to the cell, and the death is said to be accidental (Ueda and Shah, 1994). Necrosis is characterised by swelling and rupture of internal organelles, which prevents further ATP production, followed by plasma membrane lysis and the release of denatured protein, DNA fragments, lysosomal contents and other cell debris into the extracellular space (Lelli Jr. *et al.*, 1998). The cell is killed by the disruption of the ion homeostasis (Borner and Monney, 1999).

Apoptosis (programmed cell death) is a rapid, efficient and highly regulated process involved in physiological as well as pathological conditions (Verhaegen, 1998; Rizzo *et al.*, 1999). It is an essential process for development and maintenance of homeostasis during embryogenesis, cell growth and immune regulation as well as elimination of damaged, mutated or infected cells in eukaryotic organisms (Takano *et al.*, 2001).

The significance of apoptosis can be illustrated by examples of the consequences of its deregulation. An imbalance between cell death and survival is found in the pathology of a range of diseases including atherosclerosis (Geng *et al.*, 1997). Oncogenic transformation is associated with a decreased susceptibility to respond to apoptotic stimuli (Rizzo *et al.*, 1999), whereas excessive apoptosis results in acute organ failure and chronic diseases involving the loss of post-mitotic cells (Kroemer and Reed, 2000).

Apoptosis is identified by a combination of biochemical and morphological changes, which are commonly, but not uniformly, found. The inter-relationships of the constellation of features, and their precise roles, vary with stimuli and cell type.

The biochemical changes which occur are involved with the regulation of the process and mark the three phases of apoptosis: initiation or induction, decision or execution (the commitment to apoptosis) and degradation (the carrying out of apoptosis) (Kroemer and

Reed, 2000; Carmody and Cotter, 2001). Biochemical changes also alert surrounding cells to the cell's situation (Vissers *et al.*, 1999).

Most of the well-known morphological events centre around the nucleus, which shrinks, condenses and undergoes pyknosis (margination) (Alcouffe *et al.*, 1999; Yuan *et al.*, 2000). The chromatin are cleaved in a process characterised by double-stranded DNA fragmentation (Reid *et al.*, 1993A). The mitochondria and the lysosome are also involved in early events, but no physical changes are seen in these organelles until further on (Verhaegen, 1998). In the later stages, the endoplasmic reticulum is vacuolated (Reid *et al.*, 1993B) and the cell shrinks due to vesicles being formed at the endoplasmic reticulum, fusing to the plasma membrane and being released extracellularly (Samali *et al.*, 1996). This stage is seen with a scanning electron microscope as blebs, which form at the membrane (Reid *et al.*, 1993B). These become apoptotic bodies, which are later phagocytosed (Lelli Jr. *et al.*, 1998). If phagocytosis does not eventuate, a process known as secondary necrosis, in which the membranes of apoptotic bodies lyse and release their contents, as in necrosis, will occur (Skepper *et al.*, 1999).

THE INITIATION PHASE

This stage of apoptosis involves the direct effect of the stimulus. The first step in the pathway may be the engagement of a receptor at the cell surface. These receptors are called 'death receptors'. The best characterised death receptors are Fas (CD95/Apo-1), TNFR1 (p55/CD120a), DR3 (TRAMP/Apo-3), DR4, DR5 (TRAIL-2/APO-2) and DR6 (Carmody and Cotter, 2001). These interact with apoptotic machinery in the cytosol.

Fas is a member of the TNFα-receptor family, produced by $CD8^+$ T lymphocytes (Geng *et al.*, 1997). It crosslinks with anti-Fas antibody or its natural ligand FasL (Samali *et al.*, 1996). FasL is a type II membrane protein of 37kDa, and is also part of the TNFα family (Kiener *et al.*, 1997; Griffith *et al.*, 1999). Other death receptor ligands include TRAIL (TNFα-related apoptosis inducing ligand), another TNFα family member, which binds to DR4 and DR5 (Suliman *et al.*, 2001).

The ligation of FasL or other ligands results in crosslinking of the intracellular death domains of the receptors and the start of apoptotic signalling. This may occur either through

recruitment of a set of signalling proteins which activate caspase enzymes and begin an enzyme cascade (Geng *et al.*, 1997; Dai and Krantz, 1999; Artier *et al.*, 2000), or through activation of other modulators such as reactive oxygen species, glutathione efflux, ceramide or the Bcl-2 family. Both these pathways are thought to lead to changes in the mitochondria, which will result in caspase activation if this has not already occurred. The ability of the death-inducing signalling complex (DISC) to activate caspase-8 may be the deciding factor (Carmody and Cotter, 2001; Partheniou *et al.*, 2001).

It is also possible for the receptor/ligand step to be skipped, and apoptotic signalling to be directly activated by the stimulus given to the cell, if it is taken up by the cell or affects its oxidative environment.

THE EXECUTION PHASE

CASPASES

Of the fourteen caspase enzymes that have been identified, twelve are found in humans (Hampton and Orrenius, 1997; Lin *et al.*, 2000). The enzymes are called caspases based on their specificity for cleavage after aspartic residues and their active site cysteine at amino acid 163, contained in a common sequence QACRG (Kubo *et al.*, 1997; Mohr *et al.*, 1997; Vissers *et al.*, 1999). The cysteine mediates nucleophilic attack on the target substrate. It must be reduced to function and can be regulated by the glutathione ratio. The basic amino acids which surround the cysteine lower the pK_a to allow the reaction to proceed at physiological pH (Boggs *et al.*, 1998; Liu *et al.*, 2001; Hampton *et al.*, 2002B).

Caspases are present in the cytoplasm in an inactive proform with a N-terminal domain, a large subunit of around 20kDa and a small one of around 10kDa. Once apoptosis is underway, they are processed by proteases, often other caspases, to the active form, in which the large and small subunits form a heterodimer, and dimers form tetramers. A cascade is set in motion, in which the caspase enzymes are activated in a certain order (Hampton and Orrenius, 1997; Mohr *et al.*, 1997; Thornberry and Lazebnik, 1998).

There are two classes of caspase in apoptosis: one that cleaves other caspases, the initiator caspases, including caspase-1 (also known as ICE, interleukin-1β converting

enzyme), -2 (also known as Nedd-2 and ICH-1), -8 (FLICE (FADD-like ICE), MACH), -9, -10, and -13, with long prodomains, and the effector caspases that cleave other proteins and have short pro-domains. These are caspases-3 (CPP32, apopain, Yama), -6 (Mch2) and -7 (Lap3, Mch3, CMH1) (Van den Dobbelsteen *et al.*, 1996; Kubo *et al.*, 1997; Pardhasaradhi *et al.*, 1997; Stennicke and Salvesen, 1997; Thornberry and Lazebnik, 1998). Caspases-4 and -5 also have long prodomains, placing them in the initiator caspase category, but evidence for their precise role in apoptosis is still lacking, although they can cleave caspase-3 *in vitro* (Lin *et al.*, 2000).

The caspase cascades are well characterized. There are two main initiator cascades, activated by different apoptotic inducers (Borner and Monney, 1999). Caspase-8 is activated by DISC at crosslinked intracellular death domains of cell surface death receptors such as Fas or other TNFα family members, and then activates the caspases-3, -6 and -7. DISC is made up of the receptors, the receptor ligand, an adapter molecule, the Fas-associated protein with a death domain (FADD), and caspase-8 (Dai and Krantz, 1999).

Alternatively, caspase-9 is activated in the cytosol through APAF-1 (apoptotic protease activating factor-1), a 130kDa protein with an NH_2-terminal caspase recruitment domain (CARD). A complex is formed with APAF-1, cytochrome c from the mitochondria (also called APAF-2 in this situation), ATP and procaspase-9 (Liu *et al.*, 1996; Zou *et al.*, 1999; Ishisaka *et al.*, 2001). Caspase-9 will also activate the caspases-3, -6 and -7. This mechanism is mitochondrial-dependent, since cytochrome c release is required (Artier *et al.*, 2000).

The effector caspases undertake the disassembly of the cell and are therefore responsible for many of the morphological changes. The details of how this takes place are unclear, as not all substrates have been identified, and those that have been were discovered in an unsystematic fashion, so that their relationship to cell death is not always understood (Thornberry and Lazebnik, 1998; Carmody and Cotter, 2001). Since proteolysis is irreversible, effector caspase activation is seen as a point of commitment to apoptosis.

Caspases may carry out the disassembly themselves, for example through cleavage of lamin proteins, which contributes to nuclear condensation (Thornberry and Lazebnik, 1998; Zou *et al.*, 1999; Umeda *et al.*, 2001). They may also activate other proteins that execute

disassembly, such as the nuclease DFF40/CAD (Woo *et al.*, 1998; Borner and Monney, 1999; Umeda *et al.*, 2001).

The caspase enzymes also inactivate proteins that prevent apoptosis, including DFF45/ICAD, an inhibitor of DFF40/CAD, anti-apoptotic members of the Bcl-2 family and proteins involved in DNA activities such as repair (poly (ADP-ribose) polymerase, PARP, and DNA-PK_{cs}), mRNA splicing (U1-70K), mRNA processing and translocation to the cytoplasm (small nuclear ribonucleoprotein-U1, snRNP-U1) and replication (replication factor C). These cleavages have the effect of maintaining NADH and ATP levels, which, if depleted, would lead to necrosis (Sumimoto *et al.*, 1994; Palomba *et al.*, 1996; Samali *et al.*, 1996; Leist and Nicotera, 1997; Thornberry and Lazebnik, 1998; Virág *et al.*, 1998; Kroemer and Reed, 2000; Virág and Szabó, 2001).

Caspases affect cell signalling by amplifying the kinase activity of MEKK1 and that of the p38 and JNK MAPK pathways, which are pro-apoptotic (Widmann *et al.*, 1998; Aoshiba *et al.*, 1999; Umeda *et al.*, 2001). The cleavage of anti-apoptotic proteins including Raf-1, Cbl and Cbl-b and Akt shuts down the anti-apoptotic ERK and PI3K-Akt pathways (Widmann *et al.*, 1998).

THE MITOCHONDRIA

The mitochondria have been given a central position in the execution phase, as the pathways from different stimuli seem to converge there and trigger key changes, which may be committal events. These include the release of proteins, the loss of membrane potential, and the disruption of the membrane, also known as MMP, mitochondrial membrane permeability. MMP affects viability by disrupting electron transport and energy metabolism (Kroemer and Reed, 2000; Carmody and Cotter, 2001).

If the outer membrane becomes more permeable, the gradient is reduced. Proteins normally in the intermembrane space, such as some procaspases (-2, -3, -7, -9), adenylate kinase 2, heat shock proteins 10 and 60, and catabolic enzymes such as arginase sulphite oxidase move into the cytosol.

The release of cytochrome c results in a breakdown of electron flow, generating reactive oxygen species, specifically superoxide, and reducing the supply of ATP (Liu *et al.*,

1996; Cai and Jones, 1998; Green and Reed, 1998). It then triggers caspase activation as part of APAF-1 (Stridh *et al.*, 1998; Ishisaka *et al.*, 2001).

Apoptosis-inducing factor (AIF), which cleaves procaspase-3 also enters the cytosol. It is imported to the nucleus, where it induces peripheral and 'dot' chromatin condensation and DNA fragmentation (Marzo *et al.*, 2001). Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI) binds to and inhibits inhibitor of apoptosis proteins, which would otherwise sequester caspases (Cuvillier and Levade, 2001).

If the inner membrane becomes more permeable, the gradient is completely disrupted, resulting in small solutes such as Ca^{2+} and glutathione flowing out, and water and sucrose flowing in, causing swelling (Kroemer and Reed, 2000). Gradient loss may also be under redox control, as reactive oxygen species are reported to oxidise cardiolipins in the inner mitochondrial membrane, leading to disruption of the potential (Lizard *et al.*, 2000).

The mechanisms of increase of permeability and protein release, which are often thought to be linked, are debated by a number of authors. Two main processes are put forward as possibilities: the opening of a pore, and the rupture of the outer membrane.

Pore formation may occur via an ANT (in the inner membrane), VDAC (in the outer membrane) or Bax-formed channel, or by a multiprotein ensemble, called the permeability transition pore complex. This would include proteins in the inner and outer membranes at a point where the two membranes contact, such as ANT, VDAC, Bax, Bcl-2, cyclophilin D, creatine kinase or hexokinase II (Kroemer and Reed, 2000). There may be redox-sensitive sites on the pore (Lizard *et al.*, 2000; Liu *et al.*, 2001).

A pore would allow for equilibration of ions across the membranes, dissipating the gradient and uncoupling the electron transport chain, providing a plausible mechanism for that part of the process (Green and Reed, 1998). However, the pore complex suggested above would only allow movement of solutes of up to 1.5kDa, making cytochrome c and AIF too big (Carmody and Cotter, 2001).

Solving this dilemma, three mechanisms for the rupture of the outer membrane, which would release proteins, have been put forward. Pore opening may allow water and solutes to enter the matrix, causing rapid osmotic changes (Carmody and Cotter, 2001), or closure of VDAC may cause mitochondrial hyperpolarization (Suen *et al.*, 2001), or a hyperpolarization

of the inner membrane may cause the increased export of protons to the intermembrane space, an increase in osmolality and the entry of water (Green and Reed, 1998).

The lack of adequate answers for how mitochondrial changes occur is emphasised by the fact that these changes may occur in any order. Some examples show cytochrome c release and caspase activation appearing to lead to membrane potential loss, whereas other cells undergo very rapid loss of potential, which might therefore contribute to the following protein release (Cai and Jones, 1998; Green and Reed, 1998; Stridh *et al.*, 1998; Carmody and Cotter, 2001; Liu *et al.*, 2001).

THE LYSOSOME

Oxidative conditions and the build-up of substances indigestible by lysosomal enzymes promote lysosomal degradation (Hellquist *et al.*, 1997). This is a similar process to the rupture of the mitochondrial membrane, since the destabilisation of the lysosomal membrane results in the destruction of the membrane's proton gradient and leakage of hydrolytic cathespins, DNases and perhaps iron into the cytosol (Fossel *et al.*, 1994; Bolton *et al.*, 1997; Brunk *et al.*, 1997; Yuan *et al.*, 2000). Consequences of this include reactive oxygen species generation (Keller *et al.*, 1999).

In spite of the prevailing opinion of lysosomes as sturdy organelles which break down late in cell death, several studies have shown leakages of cathespin enzymes and other markers from lysosomes early in apoptosis, before mitochondrial and nuclear changes (Brunk *et al.*, 1997; Yuan *et al.*, 2000). In these cases lysosomal disruption may play a role in the initiation or commitment phases of apoptosis.

THE DEGRADATION PHASE

PHOSPHATIDYLSERINE EXPOSURE

The major classes of phospholipid are usually asymmetrically distributed across membranes. The inward-directed ATP pump aminophospholipid translocase/flippase and the outward directed pump floppase are believed to maintain the asymmetry (Kagan *et al.*, 2000).

An early feature of apoptosis, designed to mark the cells for recognition and removal by phagocytes, is the exposure of phosphatidylserine on the cell surface. Certain conditions, including ATP depletion, Ca^{2+} influx or fodrin cleavage, activate a third enzyme, a phospholipid scramblase, and inhibit the flippase, which allows for the loss of asymmetry (Kagan *et al.*, 2000; Yu *et al.*, 2000). The process is still partially unexplained. Along with phosphatidylserine, phosphatidylethanolamine is also an aminophospholipid, and so both would be expected to be exposed on the surface of the cell (Kagan *et al.*, 2000).

The involvement of oxidative stress in the pathway may explain this anomaly. In paraquat-treated 32D cells, keratinocytes with cumene hydroperoxide and HL-60 cells with a lipid-soluble peroxyl radical generator, phosphatidylserine was the only phospholipid oxidised (Kagan *et al.*, 2000). Cytochrome c, for example, has been shown to preferentially oxidise phosphatidylserineover other phospholipids (Kagan *et al.*, 2000). Furthermore, in neutrophils treated with PMA, NADPH oxidase activity was necessary for phosphatidylserine exposure, but myeloperoxidase was not. Adding hydrogen peroxide could also induce exposure (Hampton *et al.*, 2002C).

Another possibility is that phospholipid turnover, altered during apoptosis, may also contribute to the flipping of phosphatidylserine. In U937 cells, Jurkat cells and HL-60 cells, a dramatic early increase in phosphatidylserine biosynthesis is found, whereas the increase in phosphatidylethanolamine and sphingomyelin is small, and phosphatidylcholine levels decrease (Yu *et al.*, 2000).

DNA DEGRADATION

There are three parts of DNA degradation in apoptosis: single strand nicks, large DNA fragments of 50-200kbp and finally cleavage at internucleosomal regions to generate nucleosome size fragments of 180-200bp. This last stage is observed with the popular DNA laddering technique on agarose gels (originally considered the hallmark of apoptosis), and the first stage is seen with the TUNEL method. TUNEL labels DNA breaks with dUTP-biotin transferred to the free 3'-end of the cleaved DNA (Bustamente *et al.*, 1995; Samali *et al.*, 1996).

A range of Ca^{2+}/Mg^{2+}-dependent endonucleases are activated to carry out this process (Reid *et al.*, 1993A). A slight lowering of intracellular pH may be important (Pardhasaradhi *et al.*, 1997). Specific proteins identified include the nuclease DFF40/CAD, which cleaves DNA into internucleosomal fragments (Borner and Monney, 1999), DNA fragmentation factor (DFF) and p21-activated kinase 2 (PAK2) (Woo *et al.*, 1998). These are all activated by caspases. Another contributor, AIF, comes from the cytosol by a caspase-independent mechanism (Marzo *et al.*, 2001).

BLEBS: FORMATION OF APOPTOTIC BODIES

Bleb formation is related to perturbations in cytoskeletal organisation. Proposed mechanisms include a rise in cytosolic free Ca^{2+}, ATP depletion and changes in the G-actin/F-actin ratio. Since monomeric actin has four thiol groups, three of which are masked in the presence of physiological concentrations of ATP, the depletion of glutathione is also considered necessary for bleb formation (Lemasters *et al.*, 1987; Mirabelli *et al.*, 1988).

MODULATORS OF THE RESPONSE

There are many other molecular participants in the apoptotic process. Exactly how many of these are active at once is uncertain, as studies tend to measure only a few. They may, however, all be important in most circumstances. The effects of modulation are seen on caspases and the mitochondria, and may also have an effect on initiation in some cases. The role of modulators may be very fundamental, or simply a fine-tuning of the process. Their inactivation often partially prevents apoptosis. Many modulators affect others, in what can be a very complicated network of cross-regulation.

CALCIUM IONS

A rise in Ca^{2+} ions is seen to occur relatively early in apoptosis in many cell types (Escargueil-Blanc *et al.*, 1997). The ions are released by the opening of channels in the

endoplasmic reticulum by inositol triphosphate, or in an uncontrolled manner by oxidative damage to the mitochondria or endoplasmic reticulum (Halliwell and Gutteridge, 1999).

The Ca^{2+} may be involved in signalling and the opening of the mitochondrial membrane pore (Halliwell and Gutteridge, 1999). Caspase activation may indirectly require a rise in Ca^{2+} (Skepper *et al.*, 1999). Ca^{2+} also stimulates a range of Ca^{2+}-dependent enzymes including phospholipases, the endonucleases involved in DNA fragmentation and proteases such as calpains (Escargueil *et al.*, 1992).

In some cell types, depleting extracellular Ca^{2+}, buffering the intracellular Ca^{2+} or blocking the Ca^{2+} rise can prevent apoptosis (Ueda and Shah, 1994; Escargueil-Blanc *et al.*, 1997). However, Ca^{2+} influx is not always required (Sumimoto *et al.*, 1994), and in some cells, a transient elevation of Ca^{2+} by ionophores inhibits apoptosis (Lotem and Sachs, 1998; Aoshiba *et al.*, 1999).

A Ca^{2+} peak and Ca^{2+}-dependent proteases may also be features of necrosis, which can be partially blocked by protease inhibitors (Escargueil-Blanc *et al.*, 1994; Escargueil-Blanc *et al.*, 1997).

LIPIDS

Lipids may act as signalling molecules to modulate apoptosis. The neutral sphingolipid ceramide and arachidonic acid, a polyunsaturated fatty acid, can induce apoptosis (Rizzo *et al.*, 1999). Ceramide may act by disrupting the electron transport chain at cytochrome b-c1/cytochrome c (Green and Reed, 1998) or amplifying caspase-8 activity by increasing TRADD recruitment to the DISC complex (De Nadai *et al.*, 2000). It may regulate protein phosphorylation by acting directly on protein kinase Cζ (which is involved in NFκB regulation) and MAPK (Hannun and Obeid, 1995).

The activation of the transcription factors peroxisome proliferator-activated receptors (PPARs) by prostaglandins in human monocytes and macrophages results in caspase-3-mediated apoptosis, through interference with the anti-apoptotic NFκB signalling pathway, by inhibition of the transcription of its p65/RelA subunit (Chinetti *et al.*, 1998).

Lipids might also abrogate apoptosis. Sphingosine 1-phosphate can directly inhibit caspase-3, and can counteract cytochrome c and Smac/DIABLO's effects, possibly by halting

their release from mitochondria. Sphingosine 1-phosphate's generation would also reduce apoptosis as ceramide concentration would be lowered (Cuvillier and Levade, 2001).

BCL-2 FAMILY

The *bcl-2* family of genes has a role in the regulation of apoptosis. Bcl-2, Bag-1, Bcl-w, Mcl-1, A1, adenovirus E1b 19K, Ced-9 and Bcl-xl enhance survival in several cell types against different apoptotic inducers, and Bax, along with Bad, Bak, Mak, Bok, Bid, Bik, Bim and Bcl-xs have the opposing function. The ratio of protective to apoptotic proteins determines the family's overall effect (Okada *et al.*, 1998; Mizuno *et al.*, 1999; Carmody and Cotter, 2001).

The family has a similar structure to channel-forming colicins, with 7 α-helices joined by flexible loops (Green and Reed, 1998). Bax, Bid, Bim and Bak, as dimers or oligomers, form ion-conducting channels in the mitochondrial outer membrane. Their effect is reduced if they form complexes with anti-apoptotic family members. They also form pores in the membranes of the endoplasmic reticulum and nucleus. Bim, which is attached to microtubules, is released if the cell is under stress, and the others migrate from the cytosol (Zamzami *et al.*, 1995; Meilhac *et al.*, 1999; Kroemer and Reed, 2000; Carmody and Cotter, 2001). The effect of these pores may be to induce mitochondrial membrane permeability and cytochrome c release from the mitochondria, intervene in Ca^{2+} signalling by encouraging Ca^{2+} release from the endoplasmic reticulum and modulate nuclear transport (Harada *et al.*, 1997B; Meilhac *et al.*, 1999; Kroemer and Reed, 2000; Carmody and Cotter, 2001).

The family's pro-apoptotic effect is dependent to some extent on caspases. Bid is cleaved by caspase-8 to induce it to go to the membranes, and caspase-1 digests Bcl-xl, stopping its anti-apoptotic effect (Kroemer and Reed, 2000). Bcl-2 may be cleaved during apoptosis by caspase-3, going from 28kDa to a 23kDa pore-forming pro-apoptotic version (Chen *et al.*, 2000; Varela *et al.*, 2001).

As well as forming complexes, Bcl-2 is said to be an antioxidant, preventing lipid peroxidation, and being associated with an increase in intracellular GSH (Lizard *et al.*, 1998). The antioxidant properties may be indirect, since by preventing cytochrome c release and

superoxide production, change in cell redox status via GSH is reduced (Cai and Jones, 1998; Carmody and Cotter, 2001).

REDOX MODULATION

Oxidative mechanisms may have a central role in apoptosis, perhaps as a point of convergence of stimuli into a main signalling pathway. Many cell types display an increase in reactive oxygen species as part of activation, proliferation and differentiation as well as death (Lizard *et al.*, 2000). Examples of parts of apoptosis influenced by redox modulation include caspase activation, the Bcl-2 family, blebbing, phosphatidylserine exposure, lysosome degradation, death receptor activity and pore opening and gradient disruption in mitochondria.

The first indication that oxidative effects might be involved came when it was found that oxidants, such as oxLDL, caused apoptosis accompanied by increased ROS production (Dimmeler *et al.*, 1997). The idea was supported by the fact that some non-oxidative agents caused apoptosis involving oxidative stress. Examples of this type of apoptosis include that induced by TNFα, Fas, hormone and growth factor deprivation, cytokines, glucocortocoids, etoposide and epipodophyllotoxin, an inhibitor of topoisomerase II (Bustamente *et al.*, 1995; Yang *et al.*, 2000; Galán *et al.*, 2001; Takano *et al.*, 2001).

The apoptotic agents may increase ROS by upregulating endogenous oxidants, and so do not have to be oxidants themselves. The mitochondria are usually pinpointed as the key source. Others are NADPH oxidase in phagocytes, the catabolism of purine nucleotides and the metabolism of fatty acids (Takano *et al.*, 2001).

ROS, being reactive and non-specific, do not seem the ideal way to mediate the highly controlled changes of apoptosis. However, many kinases, phosphatases and transcription factors are redox responsive (Carmody and Cotter, 2001). Some selectivity can also be provided. For example, the surrounding protein environment can enhance the reactivity of hydrogen peroxide with certain cysteine residues (Hampton *et al.*, 2002B).

The induction of oxidative stress by an apoptotic agent does not prove that ROS mediate apoptosis. They may be merely a consequence of damage, rather than a cause of death (Carmody and Cotter, 2001). Further evidence for a role for ROS is given by

antioxidants that have been able, in some cases, to inhibit apoptosis. Since the oxidative burden occurs early rather than late, antioxidants are likely to affect early events (Bustamente *et al.*, 1995). Antioxidants which are able to prevent or reduce apoptosis under certain circumstances include N-acetylcysteine, GSH and vitamins E and C together, vitamin C alone, dehydroascorbic acid, γ-tocopherol, BHT, SOD and catalase (Lotem *et al.*, 1996; Niu *et al.*, 1996; Sharma *et al.*, 1996; Dimmeler *et al.*, 1997; Asmis and Wintergerst, 1998; Galle *et al.*, 1999B; Li *et al.*, 1999; Siow *et al.*, 1999; Lizard *et al.*, 2000; Takano *et al.*, 2001). Some anti-apoptotic factors, such as Bcl-2, act by reducing ROS generation (Galán *et al.*, 2001). Apoptosis sometimes occurs in the presence of antioxidants. Reactive oxygen species are therefore not a universal, essential trigger (Davis *et al.*, 2000; Galán *et al.*, 2001).

Since both ROS and antioxidants may play pro- and anti-apoptotic roles, their importance may be in the way they change, or modulate, the redox environment, which is likely to affect signalling pathways, rather than their pro- or antioxidant properties *per se*. We can, however, conclude that oxidants have a tendency to cause apoptosis, and antioxidants a tendency to prevent or delay it.

LOW DENSITY LIPOPROTEIN

Low density lipoprotein (LDL) is part of a lipoprotein system for the controlled transport and metabolism of lipids in the bloodstream. LDL is the main carrier of cholesterol, delivering it to peripheral cells. Increased levels correlate with an increased risk of atherosclerosis (Esterbauer *et al.*, 1988B; Meilhac *et al.*, 1999).

Lipoproteins are spherical structures, made up of protein embedded in a monolayer surface of polar phospholipids and cholesterol around a core of neutral cholesterol esters and triglycerides. They also contain antioxidants, including α-tocopherol, γ-tocopherol, carotenoids and ubiquinol-10 (Stocker, 1994; Meyer *et al.*, 1995). The structure is quite fluid, so the phospholipids can quickly change places (Laggner, 1987).

LDL is characterised by its density, size, M_r, chemical composition, structure and protein (Esterbauer *et al.*, 1992). LDL's buoyant density range is 1.063-1.090g/mL, and it is between 2.2-3.0 x 10^6 Daltons and 200-500Å in size (Laggner, 1987; Meyer *et al.*, 1995).

LDL has the highest proportion of cholesterol esters of the lipoproteins, making up about half its mass (Laggner, 1987). Of the fatty acids, around half are polyunsaturated fatty acids (PUFAs), mainly linoleic acid, with minor amounts of arachidonic acid and docosahexaenoic acid, although this will vary between donors (Esterbauer *et al.*, 1992).

The only protein component of LDL is apolipoprotein B100, a large lipid-binding protein of alternating α-helical and β-sheet structure. ApoB100 has 4536 amino acids and a M_r of 512,000. Details of protein folding and lipid-protein interactions are still obscure (Laggner, 1987; Meyer *et al.*, 1995).

The range of lipophilic antioxidants also varies with donor. On average, each LDL has 6 α-tocopherol molecules, $^1/_2$ γ-tocopherol, $^1/_3$ ubiquinol-10 and β-carotene (more than 20 different carotenoids have been reported), and $^1/_{10}$ oxycarotenoid (Esterbauer *et al.*, 1992).

LDL is taken up by the cell via the LDL receptor or by non-specific endocytosis (Brown and Goldstein, 1976). LDL interacts with the LDL receptor based on ionic attractions between clusters of basic amino acids and the acidic amino acids of the receptor. Divalent cations, preferably Ca^{2+}, are also required. They bind at eight sites to negatively charged residues, mostly glutamate and aspartate. Most of these sites are on apoB, although some

weak binding sites are found on phospholipids (Pifat *et al.*, 1992). LDL can also be taken up as an immune complex by Fc-gamma receptors. Autoantibodies and immune complexes have been found in patients with coronary artery disease, peripheral vascular disease and atherosclerotic lesions (Huang *et al.*, 1997).

ATHEROSCLEROSIS

Atherosclerosis is a multifactorial, complex pathological process. In most cases, it is the underlying cause of coronary artery disease (Ylä-Herttuala, 1998). It involves the accumulation of intra- and extracellular lipids (from LDL), monocyte/macrophage infiltration and foam cell formation, the proliferation of smooth muscle cells, and the accumulation of connective tissue components, forming a plaque.

Major risk factors for atherosclerosis therefore include hyperlipidemia (Nishio *et al.*, 1996) and white blood cell count. White blood cell count also predicts cardiovascular events such as myocardial infarction or thrombotic stroke, and their severity (Schmitz and Lackner, 1993; Belch, 1994).

This outline of atherosclerotic development will trace the influence of oxLDL, as well as the role of monocytes and macrophages, inflammatory conditions and antioxidants.

The first theory on the mechanism of atherogenesis was published in 1852. Rokitansky's incrustation hypothesis proposed that fibrin deposition and secondary lipid accumulation were the most important events in atherosclerosis development. In 1856, Virchow postulated lipid deposition in the arterial wall was in fact the primary event and provided morphologic evidence of inflammation. An association with monocytes, macrophages and foam cells was found in rabbit lesions in 1913, but not in humans until Stary's work in 1987. Goldstein demonstrated the macrophage scavenger receptor pathway in 1979 (Schwartz and Valente, 1995). In 1986, Ross put forward the now prevalent 'response to injury' hypothesis, an integrated model of atherogenesis involving the cells of the immune system and inflammation, as well as lipids (Jang *et al.*, 1993). The first reports on LDL were

in the 1970s. By the late 1970s and early 1980s, oxidised LDL had become a topic of discussion, and was found to inhibit growth and cause cell injury. By the end of the 1980s, oxLDL was shown to affect growth factor and cytokine production (Chisholm III and Chai, 2000).

There is still considerable debate about oxidative aspects of the theory, and many more details have been added since Ross' paper, however the basic sequence of events in atherogenesis seems to have become widely accepted.

INITIATION

The arterial lesion is initiated at the endothelium, a selectively permeable barrier between tissue and the blood, made up of a single layer of endothelial cells joined by tight junctions (Figure 2) (Lusis, 2000).

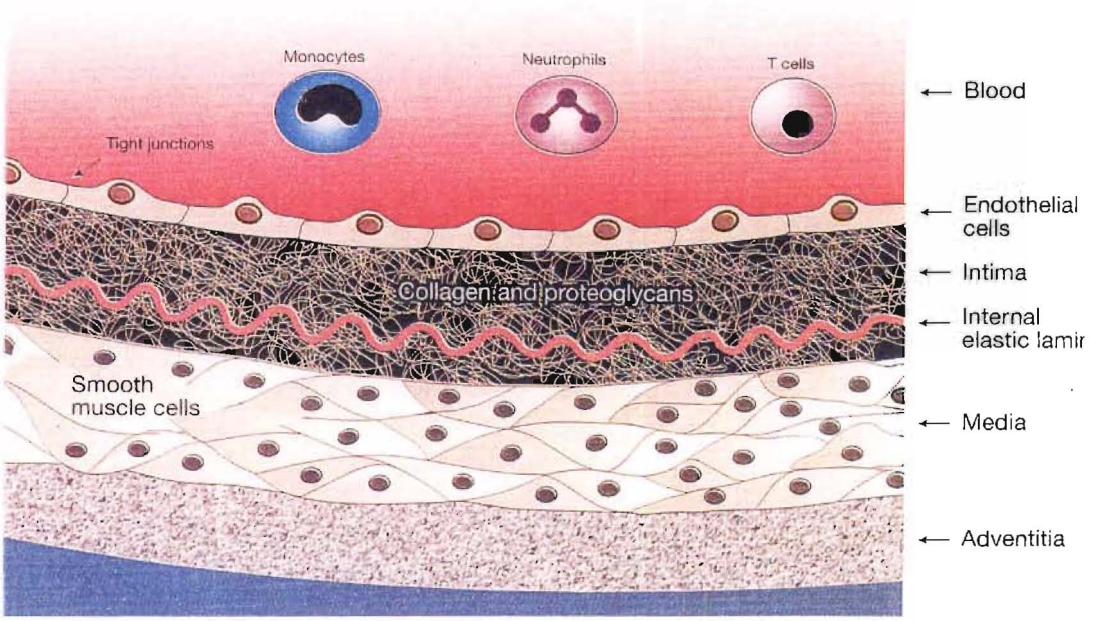


Figure 2: Structure of a normal artery. Adapted from Lusis, 2000.

The most vulnerable sites for lesion development are the inner walls of curved segments and the outer walls of bifurcations of relatively large arteries, where blood flow is likely to be disturbed by flow separation and the formation of complex secondary and recirculation flows, that is, turbulence (Wada and Karino, 2002). The disturbance in flow creates regions of low shear stress, which makes the endothelium more permeable by altering the cells' morphology and causing injury by oxidative damage, such as by superoxide (Galle *et al.*, 1999A; Halliwell and Gutteridge, 1999; Wada and Karino, 2002). This part of the artery also shows an increase in water filtration velocity, which results in sites of elevated LDL concentration (Wada and Karino, 2002) and decreased flow rate, meaning that damaging molecules such as LDL or viruses may remain there for longer, and have more of a chance to cause injury (Jang *et al.* 1993; Halliwell and Gutteridge, 1999).

If low density lipoprotein is able to diffuse through the tight junctions more easily than usual, there will be a greater chance of it becoming fixed there through interactions between the apoB molecule and the matrix proteoglycans of the subendothelium. A minimal level of oxidative modification is then likely to occur, due to its proximity to cells such as endothelial cells and smooth muscle cells, that could contribute through their oxidative waste (Steinbrecher *et al.*, 1987; Rosenfeld *et al.*, 1990; Lusis, 2000). OxLDL epitopes were found within the carotid artery wall a few hours after injection of native LDL into rat (Chisholm and Steinberg, 2000).

There is good evidence that oxLDL is present in the intima prior to the appearance of monocytes. A study by Schwenke and Carew showed an increase in oxLDL at lesion-prone sites within eight days of the start of cholesterol feeding in New Zealand white rabbits, before lesion formation began (Rosenfeld, 1996).

The alternative theory involves injury to the endothelium followed directly by monocyte migration into the subendothelial space (Jang *et al.* 1993; Halliwell and Gutteridge, 1999). Monocyte adherence is enhanced at sites of increased endothelial cell turnover, where haemodynamic injury is greater (Mitchinson *et al.*, 1996). The entry of lipoproteins into the intima would then be a secondary event.

RECRUITMENT OF MONOCYTES AND LYMPHOCYTES

The monocytes interact with the artery wall firstly by rolling arrest attachment along the endothelial surface in a process mediated by P- and E-selectins, which bind to a monocytic carbohydrate domain on L-selectin. This interaction is quite weak and sporadic. The selectins are induced by factors such as TNFα, IL-1, histamine and oxLDL (Davies, 1997; Halliwell and Gutteridge, 1999).

If the next stage of adhesion molecules is activated, the cell can flatten out and stick to the endothelial layer more tightly. Integrin $\alpha_4\beta_1$, also known as VLA-4, found on monocytes and T cells, will bind to VCAM-1 (vascular cell adhesion molecule) on the endothelium. Also LFA-1 (leucocyte function associated antigen) on leucocytes interacts with ICAM-1 and -2 (intercellular adhesion molecule) on endothelial cells. ICAM and VCAM belong to the immunoglobin gene superfamily. They are induced by cytokines such as IFNγ, chemokines, colony stimulation factors and chemotactic factors such as oxLDL (Schwartz and Valente, 1995; Davies, 1997).

The recruitment is selective, since these adhesion molecules have ligands on lymphocytes and monocytes, but not granulocytes (Hansson *et al.*, 1995). There are also very few B cells in the plaque (Huang *et al.*, 1995).

Transmigration involves adhesion molecules such as platelet endothelial cell adhesion molecule 1 (PECAM-1) on endothelial cells at intercellular junctions, and is directed by chemokines and chemotactic factors from under the endothelium, which are transported to the surface or released by the endothelium (Halliwell and Gutteridge, 1999).

Monocytes begin the process of differentiation to macrophages once in the intima, due to inflammatory activators such as IFNγ, and the presence of less than 2% serum (Schmitz and Lackner, 1993).

INFLAMMATION

The underlying cause of atherosclerosis is chronic inflammation (Galle *et al.*, 1999A). Its presence is indicated by the activation of the monocytes, macrophages and T cells that have entered the intima, along with the antigen presentation, cell-mediated toxicity and

cytokine-dependent regulatory loops that appear with it (Figure 3). OxLDL and heat shock proteins are the likely antigens. Titres of autoantibodies to hsp60 have been shown to correlate with the extent of carotid atherosclerosis (Hansson *et al.*, 1995), and both oxLDL and heat shock proteins are able to activate T cells, causing IFNγ production (Geng *et al.*, 1995). So the inflammation in the lesion results from the cells present, through the factors they produce, as well as the oxLDL.

Inflammation has been demonstrated in atherosclerosis *in vivo* through the appearance of markers such as C-reactive protein, IL-6, thrombosis tissue factor, D-dimer and neopterin, which are found to be elevated with acute coronary events (Gurfinkel *et al.*, 1999). The anti-inflammatory glucocorticoid dexamethasone suppresses atherosclerosis in experimental animals (Sakai *et al.*, 1999).

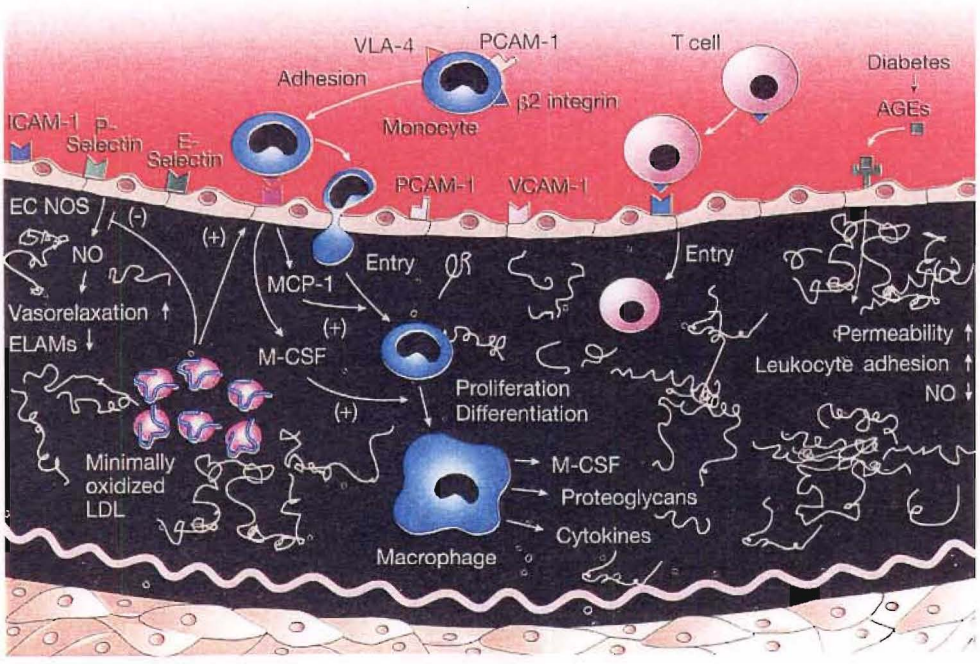


Figure 3: Inflammation in the early atherosclerotic lesion. Adapted from Lusis, 2000.

The monocytes and macrophages present in the subendothelial space will elevate their own numbers by recruiting more monocytes themselves, through the production of interleukins, TNFα and monocyte chemotactic factor. MHCII molecules will activate the new cells through T cells, exacerbating the immune response. The inflammatory response is also

increased by macrophages through further injury to the endothelium by free radicals and enzymes such as collagenase types I and IV, cathespin, heparin sulphate endoglycosidase and elastase which digest the extracellular matrix (Jang *et al.*, 1993; Schwartz and Valente, 1995).

T lymphocytes make up 10-20% of cells in the plaque, and contribute to inflammation through IFNγ. Its effects, such as smooth muscle cell expression of the HLA class II gene *HLA-DR*, have been found in plaque (Hansson *et al.*, 1989). T cells do not proliferate, so all are recruited from the bloodstream (Schmitz and Lackner, 1993). Other pro-atherogenic properties include the secretion of macrophage activating factors and the inhibition of collagen and α-actin formation, which reduces the mechanical strength of the arterial wall (Geng *et al.*, 1995).

The proinflammatory properties of oxLDL belong especially to minimally-modified LDL, such as is present early on in the plaque development (Lee *et al.*, 1999). It acts as antigen, contributing to the inflammatory immune response, and exacerbates it through its other properties (Hansson *et al.*, 1995).

OxLDL increases inflammation by the recruitment of monocytes and lymphocytes to the subendothelium by chemotaxis, and the stimulation of the surrounding endothelial cells to produce monocyte adhesion molecules and growth factors such as MCP-1 and M-CSF, which increase macrophage proliferation and accumulation (Eligini *et al.*, 1999; Shiffman *et al.*, 2000).

The growth of other cell types is also affected. Lysophosphatidylcholine (LPC) that comes into contact with human monocytes and endothelial cells increases the production of HB-EGF, a potent mitogen for smooth muscle cells and endothelial cells. LPC and oxLDL also cause human monocyte-macrophages to produce vascular endothelial growth factor (Chisholm III and Chai, 2000).

During the development of the plaque, oxLDL induces the production of inflammatory cytokines. It stimulates macrophages to release IL-8, which is chemotactic for T cells and endothelial cells, mitogenic for endothelial cells and smooth muscle cells and increases the adhesion of monocytes to endothelial cells. The highest levels were found in the macrophages from the most severe atherosclerotic plaque (Terkeltaub *et al.*, 1994; Wang *et*

al., 1996; Liu *et al.*, 1997B). OxLDL also stimulates the release of IL-1β from macrophages. This cytokine encourages proliferation of smooth muscle cells, activates helper T cells, is chemotactic for monocytes and increases vascular permeability by upregulating ICAM expression on endothelial cells (Davies, 1997). It is found at up to 60-fold its normal concentration in human atherosclerotic lesions (Ku *et al.*, 1992). Low concentrations of oxLDL also activate T cells by stimulating MHC class II-dependent production of IFNγ (Huang *et al.*, 1995).

When oxLDL is internalised by the cell, the resulting increase in intracellular oxidative stress stimulates the inflammatory response (Frostegård *et al.*, 1996; Rosenfeld, 1996). The increase in intracellular oxidative stress is likely to be responsible for many of the other changes in gene expression that oxLDL causes. NFκB, found in monocytes, macrophages, smooth muscle cells and endothelial cells in atherosclerotic lesions, is upregulated by oxidative stress and downregulated by antioxidants. It is a transcription factor that controls inflammatory responses (Suzuki *et al.*, 1994; Kaul and Forman, 1996; Flohé *et al.*, 1997; Draczynska-Lusiak *et al.*, 1998; Ginn-Pease and Whisler, 1998, Josse *et al.*, 1998; Li *et al.*, 1999), regulating TNFα, IL-1, IL-8, granulocyte and macrophage colony stimulating factors G-CWSF, M-CSF and GM-CSF, monocyte chemotactic protein, tissue factor and several adhesion molecules, most of those factors found to be regulated by oxLDL (Brand *et al.*, 1997).

OxLDL may cause oxidative stress extracellularly in the plaque by stimulating radical production. The protein moiety of oxLDL stimulates NADPH oxidase in U937 and THP-1 macrophage-like cells and polymorphonuclear leukocytes (Kopprasch *et al.*, 1998; Nguyen-Khoa *et al.*, 1999), and both oxLDL and oxLp(a) stimulate superoxide synthesis in endothelial cells (Galle *et al.*, 1999B).

OxLDL can also alter the activities of other enzymes. One example is phospholipase A_2 (type II), a Ca^{2+}-dependent enzyme that hydrolyses phospholipids at the *sn*-2 position, yielding free fatty acids and lysophospholipids. The enzyme is secreted from macrophages and found associated with them in the lipid core. It is upregulated by mildly oxidised LDL and the cytokines the oxLDL upregulates. It hydrolyses phospholipids in LDL, producing lysophosphatidylcholine (LPC) (Hurt-Camejo and Camejo, 1997; Anthonsen *et al.*, 2000).

The inflammatory conditions and the activated immune cells contribute to the further oxidation of the minimally oxidised LDL, which, as it is more extensively oxidised, takes on different properties, and continues plaque progression, in particular through foam cell formation and cell injury and death.

LDL OXIDATION

The oxidation of LDL is central to the current view of atherosclerotic development. It is commonly pinpointed as an indicator of atherosclerosis, and how easily it becomes oxidised has been linked with a donor's susceptibility to developing the disease. For example, a significant correlation was found between susceptibility of LDL to oxidation by copper and severity of coronary atherosclerosis, as assessed by angiography, in young male survivors of myocardial infarction (Regnström *et al.*, 1992). It is important to note, however, that the evidence for its oxidative function in atherosclerosis is often indirect and does not absolutely prove a causative role (Stocker, 1994).

Another factor to be aware of is that terms in the literature such as 'oxidative modification', 'minimally modified' and 'extensively oxidised' are not well defined and their definitions vary between laboratories. An attempt at definition is sometimes made through the measurement of indices of oxidation, such as TBARS and relative electrophoretic mobility.

A number of lines of evidence support the existence of oxLDL *in vivo*. Many lipid and protein products of LDL oxidation have been found in atheromas (Chisholm and Steinberg, 2000). A large proportion of lipids in the lesion are oxidised (Stocker, 1994) and early to late products of oxidation have been found, from cholesteryl hydroperoxides to aldehydes. Oxysterols and oxidised fatty acids have been found in lesions and plasma using HPLC and GC-MS techniques. They are more abundant in lesions, both fatty streaks and advanced plaques, than in normal arteries. Levels of oxysterols are high enough to be toxic to cells (Daughtery and Roselaar, 1995; Liu *et al.*, 1997A; Garcia-Cruset *et al.*, 2001).

There is little evidence of oxidation products actually within macrophage cells, as might be expected from its later accumulation there during foam cell development. This is

usually put down to the difficulty of isolating sufficient cells. However, one study found oxysterols at concentrations several-fold higher than in the plaque itself (Jessup and Kritharides, 2000).

The level of lipid oxidation found in human lesions is often proportional to the severity of the atherosclerosis (Stocker, 1994; Carpenter *et al.*, 1995B), although this is not always the case (Carpenter *et al.*, 1995A).

Lipoprotein fractions of lesions from humans and rabbits have been found to have characteristics of oxLDL (Chisholm and Steinberg, 2000) such as increased electrophoretic mobility, fragmented apoB and monocytic chemotaxis. In some studies this oxLDL is taken up by scavenger receptors, in others it is not (Steinbrecher and Lougheed, 1992; Daughtery and Roselaar, 1995).

Lipoprotein fractions of lesions are also able to react with oxLDL-directed antibodies, however care in interpretation is required in this approach, as these epitopes are also found on apoptotic cells (Chisholm and Steinberg, 2000). In five studies using poly- or monoclonal antibodies raised against forms of oxLDL or aldehyde-modified LDL and used against lesions of Watanabe heritable hyperlipidemic (WHHL) rabbits, all antibodies stained macrophage-rich regions of fatty streaks, only extracellular material in advanced plaque and nothing in normal arteries (Esterbauer *et al.*, 1992). In rabbit lesions with monoclonal antibody against the lysine-MDA adduct, quite specific for oxLDL, a positive result was found intracellularly in fatty streaks, and in diffuse and extracellular areas in more advanced plaque (Daughtery and Roselaar, 1995).

An argument against the oxidation of LDL has been the presence of antioxidants in plaque. One study of 11 human atherosclerotic advanced plaque samples found levels of ascorbic acid and α-tocopherol comparable or higher than in normal arteries. It is thought no oxidation could occur under these conditions. However, oxidation might possibly take place by processes unaffected by antioxidants, such as those carried out by enzymes or peroxynitrite. The increase in antioxidants might also be a response to oxidative attack in the plaque, not found until after the oxidation has occurred (Suarna *et al.*, 1995; Patel *et al.*, 2000).

The presence of oxLDL does not prove it is involved in the disease process. Studies with antioxidants are often used to attempt to show a causal relationship, although the oxidative stress they are blocking might have originated from a different source.

A survey of antioxidant studies on lesion development in animal models showed that 16 of 23 studies found inhibitory effects slowing lesion development by 30-50%, two reports had insignificant positive results, and the remaining five found negative results. The antioxidants used included probucol, BHT, N,N'-diphenylphenylenediamine and vitamin E (Chisholm and Steinberg, 2000).

However, a comparison of studies with individual antioxidants shows that results are very inconsistent. Both probucol and vitamin E may potentiate atherosclerosis, be beneficial against it, or have no effect in different models (Daughtery and Roselaar, 1995; Mitchinson *et al.*, 1996).

Many antioxidants also have other antiatherogenic properties which may account for their inhibition of atherosclerosis. For example, probucol inhibits monocyte adherence and decreases their release of IL-1β. Other analogues of BHT without these extra effects have had no antiatherogenic effects in WHHL rabbits (Daughtery and Roselaar, 1995). Vitamin E might inhibit the proliferation of smooth muscle cells (Esterbauer and Jürgens, 1993).

Trials in humans are limited by only being able to assess atherosclerotic development by indirect means such as mortality or morbidity, or angiographic detection of lesions. Most prevention trials show no effects of antioxidants on mortality or vascular events (Ylä-Herttuala, 1998).

Out of four vitamin E supplementation trials done on patients who already had coronary artery disease, three had no effect, and one showed a decrease in the number of infarcts, but not on total mortality. It is likely that antioxidants are unable to have much of a protective effect at this late stage of atherosclerotic development (Li *et al.*, 1999; Chisholm and Steinberg, 2000).

Several epidemiological studies have looked at antioxidant serum or plasma levels and found that populations with higher antioxidant levels are at less risk of ischemic heart disease mortality, in the case of vitamin E (Esterbauer and Jürgens, 1993), or coronary artery disease in the case of selenium (Ylä-Herttuala, 1998). Patients with angina or peripheral artery disease are likely to have lower levels of vitamin C than controls, and humans with

peripheral artery disease and coronary artery disease have low plasma levels of superoxide dismutase and thiols (Belch, 1994).

THE MECHANISM OF LDL OXIDATION

In the laboratory the process of LDL oxidation in the plaque is emulated using Cu^{2+} ions. A ratio of LDL to Cu^{2+} of 1:10-20 over 12-24 hours is required (Esterbauer *et al.*, 1992).

The Cu^{2+} binds to the LDL particle on apoB by complexing with histidine or lysine and is activated by reduction to Cu^{+}, which might be carried out by cysteine residues of apoB, pre-existing hydroperoxides, or material released by a neighbouring cell, such as thiols, superoxide anions or hydrogen peroxide (Esterbauer *et al.*, 1988B; Esterbauer and Jürgens, 1993; Gieseg and Esterbauer, 1994). Oxidation is then most likely to occur by metal-catalysed decomposition of endogenous peroxides in LDL (Jessup *et al.*, 1990). The cuprous ions break down the preexisting lipid hydroperoxides by a Fenton-type reaction to form alkoxyl radicals, which can attack PUFAs and initiate a chain reaction.

$$Cu^{+} + LOOH \rightarrow Cu^{2+} + OH^{-} + LO^{\cdot}$$

This scheme is supported by evidence that the reaction with copper is dependent upon the initial concentration of lipid hydroperoxides (Noguchi *et al.*, 1993).

The seeding peroxides may come from dietary fats, or a small amount of oxidation occurring in the bloodstream, or, if being tested *in vitro*, from the process of preparation of the LDL (Halliwell and Gutteridge, 1999). In the absence of detectable hydroperoxides, the Cu^{+} might form a transition complex with molecular oxygen, $[Cu^{2+}\text{-}O_2^{\cdot-}]$, which can also initiate oxidation or release superoxide (Esterbauer and Jürgens, 1993).

CELL-MEDIATED OXIDATION

It seems likely that the cells within the plaque, and the products they secrete, contribute to the oxidation of LDL within the intima. There are many cells present, and they have all been shown to influence this process *in vitro*.

Oxidative modification mediated by cells yields oxLDL with apparently similar characteristics to the material obtained *in vitro* using only copper ions (Jessup *et al.*, 1990). This is not surprising, as cell-mediated oxidation also requires metals in the medium in most cases, although at much lower concentrations (Kuzuya *et al.*, 1992; Meyer *et al.*, 1995). Transition metals and cells are most likely to cause oxidation via catalytic decomposition of endogenous peroxides in LDL, analogous to the process in Cu^{2+}-mediated oxidation (Jessup *et al.*, 1990). LDL prone to oxidation by Cu^{2+} is also more prone to oxidation by cell-mediated methods (Frostegård *et al.*, 1995). Hence *in vitro* oxidation represents the *in vivo* situation, as far as is currently known (Garner and Jessup, 1996).

All cell types in the lesion can oxidise LDL under the right conditions, including lymphocytes and activated platelets (Christen *et al.*, 1994; Volf *et al.*, 2000). However, neither of these, nor smooth muscle or endothelial cells, are at high concentrations in early lesions (Chisholm and Steinberg, 2000), while monocytes and macrophages are much more plentiful (Volf *et al.*, 2000).

A number of specific cell media LDL oxidation mechanisms have been proposed. Cells might accelerate oxidation by providing seeding peroxides from their own membranes to LDL (Aviram and Rosenblat, 1994; Garner and Jessup, 1996), or maintaining metals in an active state by metal reduction (Stocker, 1994). The cells may reduce metals through an oxidoreductase enzyme in a process known as transplasma membrane electron transport (Garner *et al.*, 1997B; Baoutina *et al.*, 2001B), production of superoxide and hydrogen peroxide (Chisholm III *et al.*, 1999), or thiol-recycling (Wood and Graham, 1999).

If a metal-dependent LDL oxidation *in vivo* is to be accepted, the source of the transition metal ions *in vivo* must be clarified. Many authors dispute their availability. Others, however, say that pro-oxidant iron and copper ions are present in the arterial wall (Smith *et al.*, 1992), while two separate groups claimed to find iron deposits in lesions using histochemical techniques (Garner and Jessup, 1996).

Iron and copper may be available in the acidic and oxidative conditions of an inflammatory lesion (Kuzuya *et al.*, 1992; Morgan and Leake, 1993; Winrow *et al.*, 1993; Lamb and Leake, 1994; Balagopalakrishna *et al.*, 1999; Halliwell and Gutteridge, 1999), or

reactions might be catalysed by bound metals (Garner and Jessup, 1996; Chisholm III *et al.*, 1999; Herpfer *et al.*, 2002).

Some possible mechanisms have been proposed that may account for LDL oxidation in the intima in the absence of metals. These could also play a part by augmenting any metal-dependent oxidation that may occur. They are present in the artery due to the activation of inflammation (Ylä-Herttuala, 1998).

Reactive oxygen species such as superoxide might be important. However, results of studies with SOD, catalase and NADPH oxidase have been inconclusive (Jessup *et al.*, 1993; Wilkins and Leake, 1994; Garner and Jessup, 1996; Chisholm III *et al.*, 1999). Superoxide and its derivatives may have their importance in cases of potential reactions with NO or HOCl (Garner and Jessup, 1996; Chisholm III *et al.*, 1999; Patel *et al.*, 2000).

Indirect evidence of a role for 15-lipoxygenase is provided by a range of cellular and *in vivo* studies, including transfection into cells, overexpression of the enzyme and use of inhibitors, suggesting it at least plays a contributory part in oxLDL formation (Chisholm III *et al.*, 1999; Cathcart and Folcik, 2000; Chisholm and Steinberg, 2000). 15-Lipoxygenase may act on LDL after uptake, or its products may be transferred to LDL as seeding hydroperoxides during their excretion via the CD36 receptor (Cathcart and Folcik, 2000). It might also act through the signalling properties of some of its products (Chisholm III *et al.*, 1999). The enzyme has been found in both human and rabbit lesions (Folcik *et al.*, 1997).

In vivo evidence of myeloperoxidase's participation includes the fact that 3-chlorotyrosine and hypochlorite oxidised proteins have been found in human lesions (Kopprasch *et al.*, 1998), and lesions have been found to contain material recognised by monoclonal antibodies raised against HOCl-modified LDL (Stocker, 1994).

THE PROCESS OF LDL OXIDATION AND ITS PRODUCTS

LDL oxidation has three main phases, each of which are characterised by certain processes and oxidation products. Once oxidation is initiated, the lag phase, during which lipophilic antioxidants are consumed, begins. α-Tocopherol is depleted first and β-carotene last (Esterbauer *et al.*, 1992). The lag phase is a sum of the antioxidant-dependent lag and the

autoacceleration, which is a function of the concentration of LDL and the occupancy of Cu^{2+} binding sites (Pinchuk *et al.*, 1998).

Once the antioxidants are gone, oxidation can proceed unchecked, in the propagation phase (autocatalysis), at the fastest possible rate. The acceleration is exponential, to a maximum of three molecules of lipid hydroperoxide per LDL per minute (Esterbauer *et al.*, 1992). Fatty acids, found esterified to sterol in phospholipids and triglyceride, are oxidised in this phase, primarily to lipid hydroperoxides (Steinberg, 1997).

Formed in parallel are the oxysterols, which result from cholesterol oxidation (Esterbauer *et al.*, 1992; Noguchi *et al.*, 1993; Clare *et al.*, 1995). The precursors are 7α- and 7β-hydroperoxycholesterol, found in the early stages of the plaque. By advanced plaque, it has decomposed and rearranged to form the more abundant 7-oxysterols (Brown *et al.*, 1997; Jessup and Krithrides, 2000). These 7-oxysterols, including 7α- and 7β-hydroxycholesterol and 7-ketocholesterol, make up 40% of oxysterols initially formed during oxidation. Other oxysterols include α- and β-cholestan-5,6-epoxy-3β-ol (Liu *et al.*, 1997A), cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide and cholestanetriol (Chang *et al.*, 1997; Garcia-Cruset *et al.*, 2001).

Other common oxysterols, for example 26-hydroxycholesterol (also known as 27-hydroxycholesterol), are formed by enzymatic processes. Its concentration increases with lesion severity, consistent with its production by the cytochrome P450 sterol 26-hydroxylase enzyme, which is produced by cells in response to cholesterol build-up (Carpenter *et al.*, 1995B; Liu *et al.*, 1997A; Jessup and Kritharides, 2000; Garcia-Cruset *et al.*, 2001).

The third phase of oxidation, decomposition, begins once 70-80% of the PUFAs are oxidised. The phase is characterised by a slow decrease in lipid hydroperoxide concentration, due to their labile nature, and an increase in aldehydes, fluorescent chromophores in apoB, and negative surface charge and fragmentation of apoB. LDL is not found to be in a high uptake form (leading to foam cell development) until this phase (Esterbauer *et al.*, 1992).

The hydroperoxides from PUFA oxidation are reduced to hydroxides, ketones and aldehydes, and undergo carbon-carbon bond cleavage in the presence of transition metals to form reactive short 3-9 carbon chain aldehydes. Aldehydes can also be formed by amino acid oxidation (Uchida, 2000).

Examples of the short chain aldehydes include malondialdehyde (MDA), which accounts for an estimated 20% of aldehydes formed during *in vitro* LDL oxidation, 4-hydroxy-2-*trans*-nonenal (4-HNE), also around 20% (Requena *et al.*, 1997) and hexanal, which makes up 40% of the aldehydes (Requena *et al.*, 1997). Other common aldehydes are glyoxal and the 2-alkenal acrolein (Uchida, 2000).

The aldehydes are more stable than free radicals, enabling the hydrophilic ones such as MDA to diffuse within or even escape the LDL (Uchida, 2000). They may also form adducts with apoB or other lipids, especially amino lipids, and stay associated with LDL. Lipophilic aldehydes remain with the LDL particle (Esterbauer *et al.*, 1992; Jessup and Krithrides, 2000).

Conjugation of aldehydes to apoB is an important part of the process that apoB undergoes during oxidation. The adducts most often cited are found between acrolein, 4-HNE or MDA and lysine (Uchida, 2000). 30-40% of the lysine residues are modified in this way (Requena *et al.*, 1997). The reaction occurs by Schiff base formation (shown below) or by the formation of Michael adducts with α,β-aldehydes (Esterbauer *et al.*, 1992). Lipid peroxidation products such as fatty acid epoxides, hydroxides and hydroperoxides can also cause derivatisation of apoB (Steinbrecher *et al.*, 1989).

$$\text{R-CHO} + \text{protein-NH}_3^+ \rightarrow \text{R-CH=N-protein} + \text{H}_2\text{O} + \text{H}^+$$

The amino acids most susceptible to oxidation and derivatisation are lysine (mentioned above), cysteine, and the aromatic residues tryptophan, tyrosine and histidine. 4-HNE forms adducts with histidine (Uchida, 2000) and cysteine's –SH groups, halving the number of free cysteines. Forty percent of tryptophan is oxidised through reactions with MDA or 4-HNE (Vanderyse *et al.*, 1992). These reactions neutralize the positive amino acids' charges and increase the negative surface charge of the LDL (Pifat *et al.*, 1992).

There are also other modifications to LDL during oxidation. Reactions with aldehydes can cause crosslinking and lead to covalent aggregation (Meyer *et al.*, 1995; Volf *et al.*, 2000). Alkoxyl radicals cause fragmentation, with apoB first broken into 260kDa and 232kDa fragments, and then into pieces smaller than 100kDa (Esterbauer *et al.*, 1992).

Short-chain core aldehydes derived from phosphatidylcholine are hydrolysed by lipoprotein-associated phospholipase A_2 to lysophosphatidylcholine (LPC), which increases 4.3-fold during LDL oxidation (Carpenter *et al.*, 2001).

EFFECT OF ANTIOXIDANTS ON LDL OXIDATION

Various antioxidants have been shown to be effective against LDL oxidation mediated by cells and Cu^{2+}. For example, 100μM vitamin E was able to prevent oxidation by endothelial cells as assessed by TBARS and macrophage uptake. BHT and probucol are other lipid-soluble antioxidants that were effective. Water-soluble antioxidants tested include 7,8-dihydroneopterin, 3-HAA, urate, glutathione and ascorbate, which spares other antioxidants, possibly reactivating α-tocopherol radicals to α-tocopherol. Calcium antagonists, metal chelators and platelet activating factor also have an effect (Esterbauer *et al.*, 1992; Deigner and Dresel, 1993; Gieseg and Cato, 2003).

In vivo, probucol inhibited LDL oxidation in WHHL rabbits and retarded lesion development in the aorta. Probucol was not effective in cholesterol-fed rabbits, although BHT was (Steinbrecher and Lougheed, 1992).

FOAM CELL FORMATION

Inside the subendothelium, monocytes are differentiated to tissue macrophages and become activated, contributing to LDL oxidation and inflammation in general. By the time the fatty streak has developed, most have become lipid-filled foam cells, which make up the greater part of the lesion at this stage (Figure 4) (Hegyi *et al.*, 1996).

This transformation occurs by unregulated uptake of highly oxidised LDL. Early studies concluded that smooth muscle cells made up the whole foam cell population. Macrophages were not considered to be significant until later studies on cells isolated from rabbits and monkeys found 80-90% of foam cells had characteristics of macrophages (Newman, *et al.*, 1971; Schaffner *et al.*, 1980).

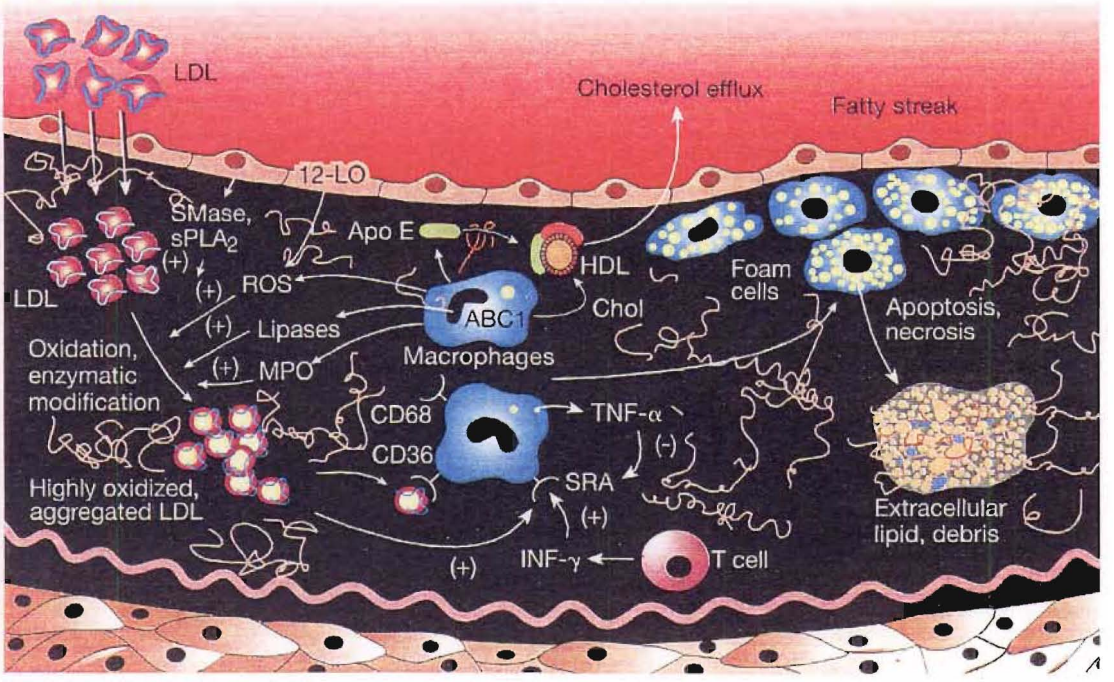


Figure 4: Foam cell formation in the atherosclerotic plaque. Adapted from Lusis, 2000.

It is probable that oxLDL is mainly taken into the cells by receptor-mediated endocytosis via scavenger receptors AI and AII, which were the first to be described (Nicholson *et al.*, 1995A). They also take up acetylated LDL. Scavenger receptors are upregulated by M-CSF, PDGF and differentiation and down-regulated by lymphokines, TNFα, transforming growth factor β, GM-CSF and LPS (Daughtery and Roselaar, 1995; Kozaki *et al.*, 1997). Isoform I is downregulated by IFNγ and consequently, fewer foam cells are found in T cell-rich regions (Grewal *et al.*, 2001). They are not regulated by increasing cellular cholesterol content (Steinbrecher *et al.*, 1987).

Modification of the lysine residues on LDL is important for recognition by the scavenger receptor (Lougheed *et al.*, 1991). Schiff bases between the epsilon amino groups of lysine and aldehydes increase the negative charge of the lipoprotein, which is responsible for interaction with the positively charged collagen domain of the scavenger receptor (Steinbrecher *et al.*, 1987; Nicholson *et al.*, 1995; Bird *et al.*, 1999). Loss of positive charge can also come from a structural change, such as fragmentation (Noguchi *et al.*, 1993).

Lipids may also have a role, as oxLDL can compete for uptake with apoptotic cells, which are recognised by phosphatidylserine (Bird *et al.*, 1999). Phospholipid aldehydes (such

as MDA and 4-HNE), as free lipids or bound to apoB, are suggested to contribute to receptor binding (Jessup and Krithrides, 2000).

More macrophage receptors that can bind and internalize oxLDL are constantly being discovered. CD36 (also known as thrombospondin receptor) and CD32 or FcγRII-B2 recognise LDL oxidised by copper but not acetylated LDL (Daughtery and Roselaar, 1995; Rosenfeld, 1996). Macrosialin/CD68 and LOX-1 (Hakamata *et al.*, 1998), SR-PSOX (Shimaoka *et al.*, 2000) and a class B scavenger receptor type I (Nicholson *et al.*, 1995A; Jessup and Krithrides, 2000) also take up oxLDL.

OxLDL can also gain access to the cell without the need for a receptor. A receptor merely adds efficiency to oxLDL's delivery (Chisholm III and Chai, 2000). Aldehydes and oxysterols, for example, can partition from the lipid phase of the oxLDL into the cell's plasma membrane (Gotoh *et al.*, 1993; Brown *et al.*, 1997). OxLDL can also be taken up by non-specific receptor-mediated endocytosis, especially in an aggregated form (Khoo *et al.*, 1988; Brown *et al.*, 1997; Hakamata *et al.*, 1998).

After endocytosis, LDL goes through the lysosomal system. The enzymes hydrolyse the cholesteryl esters and break down the protein and lipid of LDL to products that can traverse the lysosomal membrane (Maor and Aviram, 1994; Wei *et al.*, 1998).

Unesterified cholesterol and oxysterols are then transported to the membranes, where, if there is more cholesterol than the cell requires, it would normally be exported by reverse cholesterol transport (Jessup and Kritharides, 2000). However, this process is impaired in foam cells (Yancey and St Clair, 1992), and the excess cholesterol is esterified by acyl-CoA: cholesterol acyltransferase (ACAT), the activity of which oxLDL augments. It is then deposited in the cytosol as insoluble lipid droplets. The lipid droplets are responsible for the name 'foam cell' (van Reyk and Jessup, 1999). Cytoplasmic lipid droplets are mostly esterified cholesterol and some triglycerides (Naito *et al.*, 1997; Kritharides *et al.*, 1998).

Unesterified cholesterol, mainly from heavily oxidised LDL, may accumulate in lysosomes, if it is resistant to lysosomal degradation (Lougheed *et al.*, 1991; Esterbauer *et al.*, 1992; Bolton *et al.*, 1997; van Reyk and Jessup, 1999) or has inactivated lysosomal enzymes (O'Neil *et al.*, 1997; Wei *et al.*, 1998; Yuan *et al.*, 2000), and is not available to ACAT for re-esterification. This leads to crystallization and precipitation (Maor and Aviram, 1994). Free

cholesterol crystals of cholesterol monohydrate have been seen after 48 hours of cell incubation with heavily oxLDL (Kellner-Weibel *et al.*, 1998).

A progression in foam cell make-up is found in atherosclerosis. Fatty streaks contain macrophages with cholesterol ester cytoplasmic inclusions, fibrous plaques have lysosomal inclusions in macrophages and complicated lesions have smooth muscle foam cells as well (Hoff *et al.*, 1991).

EFFECT OF ANTIOXIDANTS ON FOAM CELL FORMATION

Antioxidants may also have an effect on this stage of atherosclerotic development. It was suggested vitamin E loading of LDL inhibited cholesteryl ester accumulation in J774 macrophages, by preventing cholesteryl ester formation by ACAT (Suzukawa *et al.*, 1994). However, the LDL used was not very oxidised, having only been incubated with 5μM Cu^{2+} for 90 minutes, and would not have been recognised by scavenger receptors. Another study found that loading with vitamin E before cholesterol uptake in macrophages did not affect foam cell formation (Asmis and Jelk, 2000).

FOAM CELL DEATH

Once foam cells are formed, the next change observed in the lesion is the formation of the acellular lipid core, sometimes called the necrotic core (Figure 5). This is a mass of extracellular lipid, mostly cholesterol and cholesterol esters. It will eventually have a cap of smooth muscle cells (Mitchinson *et al.*, 1996). This core is thought to come about by macrophage foam cell death (Martinet and Kockx, 2001). Evidence given for this is the presence of macrophage antigens and ceroid in the core (Mitchinson *et al.*, 1996). Ceroid is polymerized lipid and protein (Wei *et al.*, 1998), and comes predominantly from macrophages, after the crosslinking of protein of oxLDL and lysosomal proteases (O'Neil *et al.*, 1997).

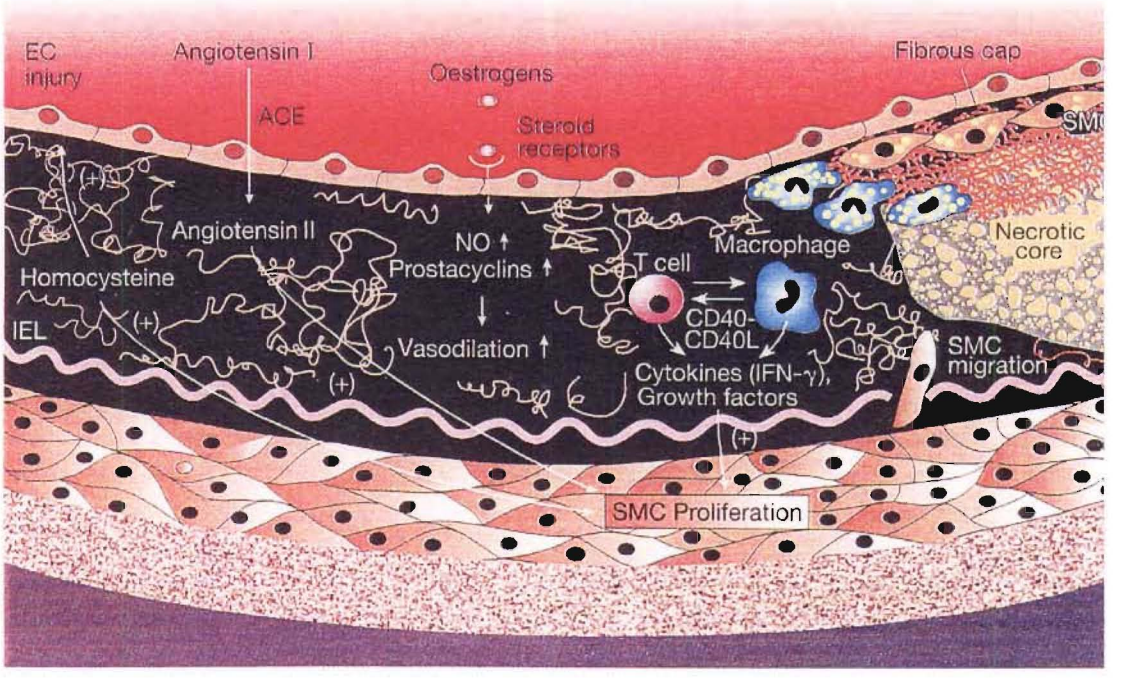


Figure 5: Foam cell death and the formation of the acellular lipid core in atherosclerosis. Adapted from Lusis, 2000.

In spite of the name 'necrotic core', it is now thought that most cells in the plaque die by apoptosis. The percentage of apoptotic cells measured in plaques of humans and animal models has been found to range from less than 1% to 60%. This variation is due to the technique commonly employed, TUNEL. It can be non-specific in tissues, labelling non-nuclear material, non-apoptotic nuclei with active gene transcription, necrotic cells and matrix vesicles formed as a result of cell disintegration. The TUNEL assay also may not differentiate between cell types, and only marks the execution phase of apoptosis which, in cell culture, takes less than six hours. As apoptosis is usually very rapid and quickly cleared by surrounding cells, lower estimates are more reasonable (Harada *et al.*, 1997A; Jovinge *et al.*, 1997; Kockx, 1998).

Only low levels of apoptosis have been detected in intimal thickened arteries or fatty streaks, with the highest levels found in advanced plaque (Kockx, 1998). Cells may undergo apoptosis in all parts of the lesion (Kockx *et al.*, 1996), but most apoptotic cells are reported to be in the core and fibrous cap, close to oxLDL immunoreactive areas (Hegyi *et al.*, 1996;

Jovinge *et al.*, 1997). Most apoptotic markers are also in regions with high densities of macrophages, rather than smooth muscle cells (Martinet and Kockx, 2001).

In spite of this, other cell types, such as endothelial cells, smooth muscle cells and lymphocytes, do undergo cell death in the lesion (Martinet and Kockx, 2001). Smooth muscle cell death is particularly important, since they form the cap of the lipid core. Apoptotic smooth muscle cells have been found in plaque, and also in ballooned rat and mouse carotid and rabbit iliac arteries after injury (Bennett and Boyle, 1998).

The apoptosis is thought to be caused by oxLDL and other factors of inflammation such as IFNγ, TNFα, IL-1β and NO and its derivatives (Mitchinson *et al.*, 1996; Bennett and Boyle, 1998; Farber *et al.*, 1999; Martinet and Kockx, 2001). OxLDL causes classic apoptosis, characterised by reduction in replication, internucleosomal DNA degradation, blebbing, fragmentation, pyknosis and chromosome condensation (Björkerud and Björkerud, 1996). OxLDL induces apoptosis in endothelial cells, lymphocytes, smooth muscle cells, macrophages, fibroblasts, lymphoblastoid cells, thymocytes, fibrosarcoma cells, and neurons (Fossel *et al.*, 1994; Björkerud and Björkerud, 1996; Chisholm III and Chai, 2000; Rusinol *et al.*, 2000).

ADVANCED PLAQUE

The American Heart Association has classified advanced plaques into three groups, depending on composition. Type IV, the atheroma, is predominantly made up of lipid core. This is the acellular collection of lipid-rich debris in the deeper part of the plaque, which forms as a result of the foam cell and non-foam cell death outlined above. Both intermediate and advanced lesions have disintegrating macrophages at the edge of the core and macrophage contents within it. Part of this lipid is ceroid, thought to be largely oxLDL released from macrophages after their death (Mitchinson *et al.*, 1995).

There are two varieties of type V. The first is calcific. The predominant mineral form is hydroxyapatite, which may be associated with elastin (Stary *et al.*, 1995). The second variety has fibrous thickening. Here the lipid core is covered by a fibrous cap and surrounded by tissues containing fibres and cells, especially macrophages and smooth muscle cells (Mitchinson *et al.*, 1995).

Due to the production of growth factors by surrounding cells, and the elimination of the growth inhibitory effect of intact endothelium, around half the smooth muscle cells in the intima experience a change in phenotype, becoming able to divide, which leads to a gradual increase in the size of the plaque, as well as fibrous cap formation (Jang *et al.*, 1993; Steinberg, 1997).

PLAQUE RUPTURE AND CLINICAL COMPLICATIONS

If the plaque ruptures, proteins of the blood's coagulation system contact the highly thrombogenic material (especially lipids and collagen) of the core (Libby *et al.*, 1998), and a blood clot, or thrombus, will form. A complicated plaque, containing hematomas or thrombi, is the third type of advanced plaque classified by the American Heart Association (Stary *et al.*, 1995). A small thrombus may simply contribute to rapid progression of the plaque, increasing its complex nature (Jang *et al.*, 1993). Three-quarters of plaques which eventually rupture and cause death are found to have ruptured previously (Virmani *et al.*, 2000).

If the clot is larger, it may lead to acute ischemic syndromes such as unstable angina, myocardial infarction or ischemic sudden death (Jang *et al.*, 1993). Ruptures are the cause of death in around 60% of sudden coronary deaths (Virmani *et al.*, 2000). This is a more frequent cause of death than obstruction, since the vessel will remodel itself to compensate for the lesion (Libby *et al.*, 1998).

Rupture often occurs at the edge of the lesion, an area rich in inflammatory foam cells and T cells, with a decreased number of smooth muscle cells. Macrophages in particular are the predominant cells in unstable areas. One way they contribute to instability is by producing inflammatory factors. A higher incidence of myocardial infarction and stroke is found during infections, during which inflammation is particularly predominant (Lusis, 2000).

Inflammatory conditions also increase apoptosis, among both the macrophages and the smooth muscle cells. Pronounced cell loss of any cell type by apoptosis increases likelihood of rupture (Kockx *et al.*, 1996). Apoptotic macrophages are greatly increased in

ruptured plaques compared to stable ones in atherectomy specimens from patients with unstable angina (Yao and Tabas, 2001).

Loss of foam cell macrophages enhances plaque instability by increasing lipid accumulation and the release of inflammatory factors as a result of their death. Fewer macrophages also means fewer apoptotic cells are able to be cleared, and more secondary necrosis occurs, amplifying the release of cellular contents and increasing the acellular lipid core (Martinet and Kockx, 2001).

The advantages of a reduction in macrophage number relate to smooth muscle cells and their formation of the new extracellular matrix and fibrous cap. Smooth muscle cells provide structural strength to the plaque through their production of proteins such as collagen (Bennett and Boyle, 1998). Without macrophages, there will be more smooth muscle cells, as the potentially damaging cells are removed (Kaikita *et al.*, 1997), and there will be less macrophage synthesis of enzymes which break down the matrix, such as metalloproteinases, collagenases, gelatinases, stromolysin and cathespins, which will also be released upon foam cell death (Bennett and Boyle, 1998; Martinet and Kockx, 2001). The presence of macrophages in a plaque therefore has both positive and negative effects.

In summary, then, monocytes and macrophages, their activation in inflammation and the oxidative stress which results, as well as oxLDL, are important components in every part of atherosclerotic development.

RESEARCH PROGRAMME

The following work will examine one particular stage of the development of atherosclerosis: the apoptosis of monocytes and macrophages caused by oxLDL, which leads to foam cell death and the growth of the acellular lipid core of the plaque. This is crucial to the clinical outcome of the plaque. Many factors may contribute to the death of the cell in this situation, and here we look in particular at the cell's ability to protect itself from the surrounding oxidative stress with its own antioxidant, 7,8-dihydroneopterin.

The effect of peroxyl radicals generated by AAPH on the cell viability of two monocyte-like cell lines, THP-1 and U937 cells, is examined in Chapter 3. Two assays, the MTT and the trypan blue assay, are used to measure changes of viability directly, and the DTNB assay looks at alterations in total cellular reduced thiol levels. 78NP is added to the cells in an attempt to protect them from cell viability and reduced thiol loss.

The same assays are used to examine the effect of oxLDL on the same two cell lines in Chapter 4, and 78NP is also included. Details of how 78NP may act as an antioxidant and how it may interact with oxLDL and the cells are then clarified. The effect of AAPH, oxLDL and 78NP on a PMA-differentiated THP-1 macrophage-like cell line is examined.

The induction of apoptosis by oxLDL in the monocyte-like cell lines is investigated in Chapter 5 using assays which examine caspase activation, phosphatidylserine exposure and changes in nuclear morphology. The effect of 78NP on these apoptotic characteristics is tested, and their relevance to a definition of apoptosis discussed.

The production of 78NP by the three cell types as a result of stimulation with IFNγ is investigated in Chapter 6. Cells are then incubated with oxLDL and IFNγ to test whether this level of 78NP is sufficient to protect the cells from cell viability loss. Finally, 78NP concentration *in vivo* is measured in samples of inflammatory material.

MATERIALS AND METHODS

MATERIALS

CHEMICALS

All reagents used in this research were of analytical grade or better. All water was deionised and ultrafiltered using a Milli-Q filtration system.

1,1,3,3-tetramethoxypropane	Sigma Chemical Co., St Louis, MO, USA
1,4-dithiothreitol (DTT)	Boehringer Mannheim GmbH, Germany
2-thiobarbituric acid (TBA)	Sigma Chemical Co.
2,2'-azobis (2-methyl-propionamidine) dihydrochloride	Aldrich Chemical Co., WI, USA
3-aminopropyltriethoxysilane	Sigma Chemical Co.
5 5'-dithiobis(2-nitrobenzoic acid)	Sigma Chemical Co.
7,8-dihydroneopterin	Schricks Laboratory, Switzerland
Acetic acid (glacial)	BDH Lab Supplies, Poole, England
Acetone	BDH Lab Supplies
Acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin	Sigma Chemical Co.
Apotest-FITC A700 kit	Nexins Research, The Netherlands
Argon gas	BOC Gases, New Zealand
Ascorbic acid	Sigma Chemical Co.
BCA protein determination kit	Pierce, Illinois, USA
Bovine serum albumin	Gibco BRL, N.Y., USA
Butylated hydroxytoluene	Sigma Chemical Co.
CHAPS	Sigma Chemical Co.
Chelex 20 resin	Bio Rad Laboratories, USA
Chelex 100 resin	Bio Rad Laboratories
Chloroform	BDH Lab Supplies
Cholesterol reagent	Roche Diagnostics, USA
Copper (II) sulphate 5-hydrate	BDH Lab Supplies
Dimethyl sulphoxide (DMSO)	Sigma Chemical Co.
Dialysis tubing	Biolab Scientific, New Zealand
Diammonium hydrogen orthophosphate	BDH Lab Supplies
Earle's Balanced Salts solution	Sigma Chemical Co.
Ethanol	Ajax Laboratory Chemicals, Australia
Ethylene diamine tetracetic acid	Boehringer Mannheim GmbH
Eukitt mounting medium	Electron Microscopy Sciences, PA, USA
Ferrous ammonium sulphate	Sigma Chemical Co.
Fetal bovine serum	Gibco BRL, New Zealand
Griess reagent (Modified)	Sigma Chemical Co.
HEPES	Sigma Chemical Co.
Hoechst 33342	Sigma Chemical Co.
Hydrochloric acid	BDH Lab Supplies
Iodine	BDH Lab Supplies
Interferon-γ (specific activity $2x10^7$mg/protein)	Boehringer Mannheim GmbH
Lipoprotein Electrophoresis kit	Beckman Coulter, USA
Neopterin	Schricks Laboratory
Methanol	BDH Lab Supplies
MTT	Sigma Chemical Co.

Nitrogen gas	BOC Gases
Orthophosphoric acid 85%	BDH Lab Supplies
Paraformaldehyde	BDH Lab Supplies
Penicillin/Streptomycin (1000U/mL penicillin G and 1000µg/mL streptomycin)	Gibco BRL, N.Y., USA
Phorbol 12-myristate 13-acetate	Sigma Chemical Co.
Potassium bromide	BDH Lab Supplies
Potassium iodide	May and Barker Ltd, England
RPMI 1640	Sigma Chemical Co.
Sodium chloride	BDH Lab Supplies
Sodium dihydrogen orthophosphate	BDH Lab Supplies
Sodium dodecyl sulphate	Sigma Chemical Co.
Sodium hydroxide	BDH Lab Supplies
Sodium nitrite	May and Baker Ltd
Sucrose	Chelsea Sugar Refinery, Auckland
Sulphuric acid (18.44M)	BDH Lab Supplies
Tergitol, type NP-40	Sigma Chemical Co.
Trichloroacetic acid	Sigma Chemical Co.
Trypan blue solution (0.4%)	Sigma Chemical Co.
Xylenol orange	Sigma Chemical Co.

SOLUTIONS, BUFFERS AND MEDIA

A. LDL PREPARATION SOLUTIONS

EDTA FOR BLOOD COLLECTION

A 100mg/mL EDTA solution was prepared by dissolving EDTA in nanopure water and adjusting the pH to 7.4 with 10M NaOH.

DENSITY GRADIENT SOLUTIONS

The required amount of NaCl was dissolved in nanopure water, to give densities of 1.08g/mL, 1.05g/mL and 1.00g/mL. A 10mg/mL EDTA solution was prepared as above and added to each density gradient solution to give a final concentration of 1.0mg/mL.

PHOSPHATE BUFFERED SALINE

PBS was prepared from stock solutions of NaCl and NaH_2PO_4 pH 7.4 to give final concentrations of 150mM and 10mM respectively. Before use, it was stirred overnight with washed with chelex-20, filtered, and bubbled with nitrogen gas for 10 minutes.

LIPOPROTEIN GEL ELECTROPHORESIS SOLUTIONS

B-2 barbital buffer at pH 8.6 was made by dissolving the contents of the buffer bottle in 1500mL nanopure water. The lipoprotein working stain was made by mixing 3mL of concentrate with 165mL ethanol and 135mL nanopure water. The fixative was made by adding 90mL nanopure water to 450mL reagent alcohol and 30mL glacial acetic acid and mixing thoroughly. Destain solution was a mixture of 450mL ethanol and 550mL nanopure water.

All solutions were stored at room temperature.

B. SOLUTIONS FOR CELL EXPERIMENTS AND CULTURE

MEDIA FOR CELL CULTURE

Media was prepared from the powder according to the manufacturer's instructions and filtered using a Millex-GP50 filter unit of 0.22μm pore size from Millipore (Molsheim, SA) and a CP-600 pump from Life Technologies, into sterilised bottles. It was stored at 4°C, but warmed to 37°C in a waterbath before use.

MEDIA FOR CELL EXPERIMENTS

RPMI with phenol red and L-glutamine was supplemented with 10,000U/mL of penicillin/streptomycin and 5% heat inactivated fetal bovine serum.

PBS was prepared as above and autoclaved (15 minutes, 121°C, 15psi). It was stored at 4°C and warmed before use.

RPMI 1640 with L-glutamine but without phenol red and sodium bicarbonate, and Earle's Balanced Salts solution without phenol red or sodium bicarbonate were prepared as outlined above.

PHORBOL 12-MYRISTATE 13-ACETATE

A stock solution of PMA was made by dissolving PMA in DMSO to give a final concentration of 0.1mg/mL. This was stored at –20°C, and diluted to 100ng/mL in RPMI 1640 media before addition to the cells.

7,8-DIHYDRONEOPTERIN SOLUTION

7,8-Dihydroneopterin (2.552mg) was measured out using a Mettler Toledo UMX2 microbalance (Mettler Toledo GmbH, Greifensee, Switzerland), and dissolved while on ice in a foil-covered bottle containing N_2-bubbled PBS to a concentration of 2.0mM. Before addition to cells, the solution was sterilised through a 0.2μm filter. 78NP solution was prepared fresh each day.

C. SOLUTIONS FOR 78NP AND NEOPTERIN HPLC ANALYSES

A 150mM neopterin standard was made up by dissolving neopterin in 10mM phosphoric acid through stirring and sonication in the dark. It was aliquoted out and stored at –20°C.

The mobile phase was prepared on the day of analysis and consisted of a 20mM ammonium phosphate solution containing 5% methanol at pH 6. The mobile phase was filtered through a 0.45μM filter before use.

The acidic iodide solution was made by dissolving 2.7g solid iodine and 5.4g potassium iodide in 35mL of nanopure water. 3.71mL of 50% TCA was then added, and the final volume made up to 50mL.

D. SOLUTIONS FOR PUS PREPARATION

20mg/mL BHT was made up in methanol and stored at 4°C.

E. SOLUTIONS FOR HOECHST STAINING

Two percent 3-aminopropyltriethoxysilane was made by diluting a 98% solution in acetone.

A 4% paraformaldehyde solution was made by adding paraformaldehyde to water, heating to 60°C, and adding a few drops of 1M NaOH to help it dissolve. After cooling to room temperature, the solution was diluted with PBS. It was stored at –20°C.

Hoechst 33342 stain was diluted to a working concentration of 10μg/mL on the day of use in PBS. Stock stain was stored at -20°C.

F. SOLUTIONS FOR MTT ASSAY

MTT powder was dissolved in RPMI 1640 without phenol red to 5mg/mL, sterilised through a 0.2μm filter and stored at –20°C for up to 2 weeks in the dark.

A 0.01M HCl solution was made up from 11.44M HCl and nanopure water, and stirred slowly with 10% SDS.

G. SOLUTIONS FOR DTNB THIOL ASSAY

DTNB made up to 3mM in 10mM NaH_2PO_4 buffer, stored at 4°C in tinfoil, and used within 2 weeks.

H. SOLUTIONS FOR CASPASE ACTIVATION ASSAY

Caspase buffer was prepared by dissolving sucrose, CHAPS and Tergitol NP-40 to final concentrations of 10%, 0.1% and 10^{-4}% respectively in a 100mM HEPES solution. The pH was adjusted to 7.25, and the buffer stored in 5mL aliquots at –20°C.

1M DTT was made up on the day of analysis in water, and kept it in the dark on ice.

DEVD-AMC, the caspase substrate, was dissolved in 100% DMSO by adding DMSO straight to the bottle the substrate was supplied in and swirling. It was stored in small aliquots at –20°C until required and refrozen no more than twice.

I. SOLUTIONS FOR FOX ASSAY

A 25mM solution of sulphuric acid was made from a 1M stock solution and replaced weekly, due to the leaching of ions from the glass bottle.

The 5mM xylenol orange solution and 5mM ferrous ammonium sulphate were made up in the 25mM sulphuric acid.

72% TCA was made by dissolving 72g in nanopure water made up to a final volume of 100mL and was stored at 4°C.

Methanol/chloroform and acetic acid/water mixtures were made up in a 1:1 ratio.

J. SOLUTIONS FOR NITRITE ANALYSIS

Griess reagent was dissolved in nitrogen-gassed nanopure water to give a working concentration of 40mg/mL.

K. SOLUTIONS FOR TBARS-HPLC LIPID ANALYSIS

The MDA standard was made by diluting a 6.07M 1,1,3,3-tetramethoxypropane solution in a 40:60 ethanol/water mix, then diluting further with water to the required concentration.

The mobile phase was a 100mM sodium dihydrogen orthophosphate solution, which was adjusted to pH 6.8 with 10M sodium hydroxide. After filtration, 35% HPLC grade methanol was added, and the final mixture degassed by sonication.

A 42mM 2-thiobarbituric acid solution was prepared fresh on the day of analysis in nanopure water by stirring on a hot plate.

A 20mg/mL BHT solution was made up in methanol and stored in the fridge.

METHODS

LDL PREPARATION

A. BLOOD COLLECTION AND PLASMA PREPARATION

Two hundred mL of blood were drawn from each donor by venipuncture using a 15G needle and line after an overnight fast. The blood was collected directly into 50mL Falcon tubes containing 0.5mL of 10% EDTA, to give a final concentration of 0.1% EDTA. The tubes were centrifuged at 4,100g for 20 minutes at 4°C to separate the plasma from the cells.

The plasma was then transferred to SS34 rotor centrifuge tubes, and centrifuged at 11,000g for 30 minutes at 4°C in the fixed rotor. The plasma was collected into a single measuring cylinder, to which sucrose was added to give a final concentration of 0.6% sucrose before storage at –80°C in 20mL aliquots. Plasma was stored for no longer than 4 months before use.

B. EXTRACTION OF LDL FROM PLASMA

A 20mL tube of frozen plasma was defrosted under cold running water, and then centrifuged at 4,100g for 10 minutes at 4°C to pellet precipitated fibrinogen. The supernatant was decanted into a beaker, and KBr gradually dissolved in it to a final concentration of 410mg KBr/mL plasma. The resulting solution was pipetted into ultracentrifuge tubes, 3.5mL per tube.

Using a specially manufactured right-angled needle, NaCl/EDTA solutions of decreasing densities were layered on top of the plasma. Tubes were then placed in the Beckman 41-Ti rotor in the Beckman L8-70M UH ultracentrifuge (Beckman Coulter, CA, USA) for 22 hours, at 40,000rpm and 10°C.

At the end of this time, the tubes were removed from the rotor, and the VLDL layer taken off. The orange LDL layer was then sucked out using a right-angled needle attached to a syringe. The LDL was degassed with argon gas and filter sterilised into a

sterile 5mL serology tube. The purified LDL was stored at 4°C in the dark for a maximum of two weeks.

C. DIALYSIS

The LDL solution was dialysed to remove residual EDTA for 18 hours against three changes of the prepared PBS at 4°C.

D. CHOLESTEROL DETERMINATION

LDL concentration was determined as a function of cholesterol level using a kit supplied by Boehringer Mannheim.

One mL of cholesterol reagent was added to 10μL of LDL and incubated at room temperature for 20 minutes, after which the absorbance was measured at 500nm against a blank containing only cholesterol reagent. The concentration of the LDL was calculated assuming 31.64% of the LDL particle is cholesterol by weight, and that the molecular weight of LDL is 2.5 MDa (Gieseg and Esterbauer, 1994).

E. LDL OXIDATION

LDL to be oxidised was filtered with Acrodisc 0.2μm Supor membrane sterile filters (Pall Gelman Laboratory, MI, USA) and incubated at 37°C for 24 hours with a final concentration of 50μM $CuSO_4$. The $CuSO_4$ came from a 100mM stock solution, sterilised with a 0.2μm filter before addition to LDL. The LDL was then stirred with chelex-100 on a rotational mixer for two hours at 4°C to remove Cu^{2+} ions.

F. LDL CONCENTRATION

Both oxidised and non-oxidised LDL was concentrated five-fold before incubation with cells, using Urifil-10 concentrators (Millipore, MA, USA). The LDL was then sterilised with an Acrodisc 0.2μm HT Tuffryn membrane sterile filter from Pall Gelman Laboratory and the tube stored in the dark at 4°C for up to two weeks.

G. LIPOPROTEIN GEL ELECTROPHORESIS

Electrophoretic mobility was determined with a Beckman Paragon native gel electrophoresis kit and apparatus according to the manufacturer's instructions.

Three 3μL applications of native or oxidised LDL were applied per lane, allowing time for absorption between each application. The gel was electrophoresed for 30 minutes at 100 volts in B-2 Barbital buffer. The gel was then placed in fixative solution and dried overnight. The gel was stained for five minutes in lipoprotein working stain and destained by dipping it in the destain solution followed by immersion for five minutes.

CELL CULTURE

Cells were grown in culture flasks in 50mL solutions of RPMI 1640 with phenol red and L-glutamine, containing 5% HIFBS and 10,000U/mL penicillin/streptomycin. Cells were not allowed to grow to a concentration of over 1 million/mL. They were maintained in an incubator (Precision Scientific, Illinois USA) at 37°C, in a humidified atmosphere containing 5% CO_2. Cells were counted using a haemocytometer and a light microscope.

CELL EXPERIMENT PROCEDURES

All manipulations were performed under aseptic conditions in a Class II biological safety cabinet (Clyde-Apac BH 2000). All instruments and equipment were either sterile plasticware (Nunc products, Nalge Nunc International, Falcon products, Bector Dickinson and Co. or Sarstedt, NC, USA) or had been sterilised by autoclave (15 minutes, 121°C, 15psi).

If differentiation of THP-1 cells was required, cells were counted and the required number resuspended in phenol red RPMI 1640 containing HIFBS and PS as well as PMA to a concentration of 100ng/mL. Cells were plated out at 1 $x10^5$ cells per well, and incubated for a week before use in experiments. The media was changed every three days.

For experiments with non-adherent cells, wells of plates were coated with 10μL of 5% BSA per well, to prevent the cells adhering to the plastic in the absence of serum. Cells were counted and the required amount was washed twice with PBS and resuspended in warm RPMI 1640 (without phenol red), or Earles' Balanced Salts Solution, to a concentration of 25 x 10^5/mL. The cells were aliquoted out into wells containing RPMI 1640 or EBSS to give a final concentration of 5 x 10^5/mL.

If interferon-γ was to be included, it was added to the well 10 minutes before the cells. If 78NP was to be present it was added after the cells 15 minutes before any oxidant. This 15 minute incubation took place in the dark. After the oxLDL, AAPH or EtOH were added, plates were put into the incubator for the duration of the experiment.

CELL VIABILITY ANALYSIS BY MTT ASSAY

The MTT assay is based on the method described in Mosmann's paper of 1983. The tetrazolium salt MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, is cleaved by reduction to a coloured formazan end product. This requires metabolically active cells. The addition of glucose to the media, or the enhancement of glucose uptake by cells, will increase MTT reduction (Takahashi, 2002). The absorbance of the dissolved formazan was found to be proportional to the number of cells present in all cell types tested by Mosmann.

The MTT is taken up by endocytosis, since it is not permeable to the lipid membrane, and is reduced by electron donors from a range of organelles (Takahashi, 2002). Both enzymes of the mitochondria and the endoplasmic reticulum are likely to be responsible, although from inhibitor tests, NADH and NADPH appear to contribute more to the reduction than succinate, as was previously believed (Berridge, 1996). Glycolysis is therefore a more important process than respiration in determining the level of MTT reduction.

Wellplates were coated with BSA as described in the above section. Cells from the experiment were washed twice with warm sterile PBS, then resuspended in 100μL RPMI 1640, and aliquoted into the wells, which already contained 900μL non-phenol red RPMI 1640. 100μL MTT reagent was added, and the plates incubated for 3 hours at 37°C. The

resulting purple crystals were dissolved by adding 0.5mL of SDS-HCl and mixing with a pipette tip. Absorbance was measured at 570nm, using water as the blank.

CELL VIABILITY ANALYSIS BY TRYPAN BLUE EXCLUSION ASSAY

This technique uses trypan blue, a blue dye able to enter cells once the integrity of the cell membrane has been breached and the cells are no longer able to pump the dye out. It therefore allows a visual assessment of the state of the membrane.

20μL of cell sample was taken from a well and mixed with 5μL of trypan blue dye (to a final concentration of 0.08%). The colour was allowed to develop for 20 seconds, and then the cell suspension was placed on a haemocytometer and examined by microscopy. Live cells appeared opaque and dead cells blue. The cell viability was calculated as a percentage by dividing the total number of viable cells by the total number of both dead and viable cells.

THIOL MEASUREMENT BY DTNB ASSAY

This assay is based on the method published by Boyne and Ellman in 1972 using 5 5'-dithiobis (2-nitrobenzoic acid) (DTNB) or Ellman's reagent. DTNB is an aromatic disulphide, which is reduced by thiol-disulphide exchange to form the highly coloured thiophenol anion, 3-carboxylato-4-nitrothiophenolate (CNTP) at a stoichiometry of one anion per biological thiol originally present (Ando *et al.*, 1973; Jocelyn, 1987). The technique as presented here measures total thiols, including those which form part of protein, and non-protein thiols, such as glutathione (Hu, 1994).

Cell incubations with oxidant were carried out in 6-well plates with 3mL of cell suspension in each. After the allotted time, cells were removed from wells and centrifuged for 5 minutes at 430g at 4°C, then washed twice in cold PBS. The pellet was resuspended in 1mL PBS, and the cells lysed by sonication for 2 minutes.

0.5mL 10% SDS and 15μL DTNB (final concentration 30μM) were added and the solution was mixed and incubated on a rotational mixer for 30 minutes at room

temperature. The SDS was used to denature the proteins, in order to ensure the DTNB had free access to the thiols (Soszynski *et al.*, 1997). The resulting colour was measured spectrophotometrically at 412nm. The concentration of total cellular thiols could be calculated using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}.

CASPASE ACTIVATION ASSAY

DEVD-AMC, Ac-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin), mimics the target sequence of caspase-3 and other group II effector caspases, and determines if the caspase enzymes are activated (Stridh *et al.*, 1998).

After incubation with oxLDL or ethanol, cells were pelleted by centrifugation at 5000rpm for 5 minutes at room temperature. The cell pellets were resuspended in 1mL cold PBS, aliquoted into 10^5 cell portions and centrifuged again. The supernatant was taken off, and the pellet frozen at –80°C for a maximum of two weeks. The assay was carried out on a Hitachi fluorometer in a custom-made black plastic cuvette, at a constant temperature of 37°C.

The working solution of 1mL buffer, 5µL of a 1M DTT solution (final concentration 5mM) and 1µL of caspase substrate (final concentration 50µM) was mixed just before the assay and kept warm in the dark. 100µL was added to each pellet and mixed, and the increase in fluorescence over time at λ_{ex} 370nm (band width 5nm) and λ_{em} 445nm (band width 10nm) was monitored for 3 minutes per sample. The rate at which this occurs was described as 'caspase activity'.

ANNEXIN V-FITC AND PROPIDIUM IODIDE ASSAY

Annexin V is a Ca^{2+}-dependent endogenous protein that preferentially binds to membrane-associated phosphatidylserine. The level of binding is measured through conjugation to fluorescein-isothiocyanate (FITC). Propidium iodide is a dye that enters the cells with damaged plasma membranes and incorporates itself into DNA (Blankenberg *et al.*, 1999; Farber *et al.*, 1999).

After treatment with oxLDL or ethanol, and washing with PBS, 10^5 cells were aliquoted into capped plastic tubes and spun at 500g for 2 minutes. The supernatant was then aspirated off. To the cell pellet, 2μg of PI solution, 200μL of binding buffer (diluted from 10x) and 2μL Annexin-V-FITC (diluted from 1000x) were added, according to the manufacturer's instructions (Apotest-FITC A700 kit, Nexins Research). The tubes were left in the dark for 10 minutes, and the fluorescence detected by bivariate flow cytometry with a Vantage fluorescence-activated cell sorter from Becton Dickinson.

HOECHST STAIN

The fluorochrome Hoechst 33342 binds specifically to A-T base pairs in DNA (Schobersberger *et al.*, 1996).

After incubation for 24 hours with oxLDL or ethanol, cells were washed with PBS, and a pellet of 10^5 cells was resuspended in 60μL of 4% paraformaldehyde in PBS and left on a rocker at 4°C for 30 minutes. A 20μL drop of cell suspension was put on a slide treated with 2% 3-aminopropyltriethoxysilane.

After the cells had dried, the slides were washed twice in PBS and stained with one drop of Hoechst at 10μg/mL for 10 minutes in the dark. Slides were then washed five times in distilled water, and after drying, a coverslip was applied using Eukitt mounting medium.

Cells were examined using a Zeiss Axioskop 2 MOT epifluorescent microscope (Carl Zeiss (NZ) Ltd, Wellington, New Zealand), fitted with an HBO 100W mercury vapour lamp and Axiovision 3.1 software. The fluorescent filter used was Chroma Technology Corp. Filter set 82000 single band UV exciter for DAPI/Hoechst/AMCA (Excitation filter D360/40x, Beamsplitter 82100, Emission filter 82101). The images were captured using a Zeiss Axiocam HRc CCD camera and processed with Adobe Photoshop 5.0.

GRIESS REAGENT ASSAY

Griess reagent contains 1% sulphonamide, 0.1% naphthylenediamine dihydrochloride and 2.5% phosphoric acid (Levine *et al.*, 1996; Rajora *et al.*, 1996). It is used to measure NO_2^- as an endpoint of NO production, after its oxidation in aqueous solution (Halliwell and Gutteridge, 1999).

$$4NO^{\cdot} + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

Cells were incubated with or without 300U/mL interferon-γ for up to 48 hours. After centrifugation for 5 minutes at 1500rpm, the supernatant was removed. The cells were then washed twice in PBS, resuspended in water, and sonicated to ensure lysis. Equal volumes of test solution from cells or supernatant and Griess solution were mixed, and the absorbance measured at 540nm after 15 minutes. The concentration of nitrite was determined using a standard curve constructed with sodium nitrite.

PROTEIN DETERMINATION

Total cell or pus protein concentration was determined using the bincinchonic acid (BCA) estimation method with a kit supplied by Pierce, Illinois, USA.

The working reagent was freshly prepared by mixing reagents A and B in a 1:50 ratio:

Reagent A: sodium carbonate, sodium bicarbonate, BCA detection reagent, sodium tartratein, 0.1N sodium hydroxide.
Reagent B: 4% $CuSO_4.5H_2O$

The assay was carried out by incubation of 50μL samples with 1mL working reagent at 60°C for 30 minutes with gentle shaking. Solutions were then cooled in water to stop the reaction before reading the absorbance at 562nm against a water blank. The protein concentration was calculated from a standard curve constructed using BSA.

TEST FOR LEVEL OF AGGREGATION IN LDL

Aggregation levels in LDL can be found by spectrophotometric analysis of turbidity at 680nm (Maor *et al.*, 1997; Llorente-Cortés *et al.*, 2000).

Both native and oxidised LDL were diluted to 0.5mg/mL and placed into plastic cuvettes. The absorbance was then measured on the spectrophotometer.

FOX ASSAY (FOR PROTEIN)

The Ferric-Xylenol orange (FOX) assay modified by Gebicki (Gay *et al.*, 1999) was used to measure protein hydroperoxide (ROOH) formation in LDL.

This assay measures protein hydroperoxide levels by their oxidation of Fe^{2+} ions to Fe^{3+} ions. The Fe^{3+} ions then react with xylenol orange (XO) to form a complex which can be measured spectrophotometrically at 560nm.

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + OH^{-} + RO^{\cdot}$$

$$Fe^{3+} + XO \rightarrow Fe\text{-}XO$$

One mL of LDL solution was mixed with 140µL of cold 72% TCA by vortexing, and chilled on ice for 5 minutes for protein precipitation. The proteins were then pelleted by centrifugation at 15,000rpm and 4°C for 5 minutes. The supernatant was removed, and the pellet resuspended in 1mL of a 1:1 methanol/chloroform mixture and recentrifuged. The supernatant was again removed, and the tube dried upside down for five minutes. The pellet was then dissolved in 900µL of a cold 1:1 acetic acid/water solution.

To the test tubes, and also three control tubes containing acetic acid and water, 50µL xylenol orange and ferrous ammonium sulphate were added, and the mixtures were incubated for 30 minutes in the dark. After centrifugation at 15,000rpm for 5 minutes to remove debris, the absorbance could be read at 560nm using a water blank, and the concentration of peroxide calculated using an extinction coefficient of $48{,}000M^{-1}.cm^{-1}$.

PTERIN DETERMINATION USING HPLC

Cell culture media samples were transferred to 1.5mL centrifuge tubes for analysis. Cell lysates were prepared by washing the cells twice in PBS, then resuspending in nanopure water and sonicating for 5 minutes. Adherent cells were sonicated in their wells for 10 minutes.

Twenty μL of acidic iodide solution (5.4% I_2/10.8% KI) and 25μL 50% TCA were added to a 250μL sample, mixed and incubated in the dark for 60 minutes. If the 78NP was to be measured unoxidised, the iodide solution was not added. The oxidation allows both 78NP and neopterin to be measured.

A spatula of ascorbate was then added to oxidise the iodine and samples were centrifuged at 8800g for 15 minutes.

Five μL of 2M ammonium phosphate was added to 100μL of the supernatant and the treated sample was transferred to glass autosampling vials for analysis on a Shimadzu (New Zealand, Ltd) SIL-10A automated high performance liquid chromatography system equipped with a SIL-10A autoinjector, DGU-14A pump, RF-10Axl fluorescence detector and L-ECD-6A electrochemical detector with a glass carbon electrode and silver chloride reference cell. 10μL injections were passed through a RP-C18 4.6 x 250mm column (Phenomenex) maintained at 35°C inside a Shimadzu CTO-10A column oven. The mobile phase (20mM $NH_4H_2PO_4$, pH 6.0 and 5% methanol) was pumped through the system at a flow rate of 1mL/min.

Neopterin was measured using the fluorescence detector set at an excitation of 353nm and emission of 438nm. 78NP was detected using the ECD detector with the electric potential set to +0.6V. Peak areas were calculated using the Shimadzu software package, by comparison with standards.

TBARS-HPLC LIPID ANALYSIS

This HPLC technique is used to quantify lipid peroxidation. Malondialdehyde (MDA), a breakdown product of lipid peroxides, reacts with 2-thiobarbituric acid (TBA) to form a TBA-MDA adduct, a pink colour which can be measured by fluorescence

detection. MDA is unstable so is prepared immediately before use by hydrolysis of its derivative 1,1,3,3-tetramethoxypropane. The assay used here is a modification of the standard HPLC method described by Draper *et al.* in 1993.

The amount of free MDA produced in most peroxidizing lipid systems is too low to give measurable colour. The level detected is formed by the decomposition of hydroperoxides during the acid heating stage. The decomposition can also result in peroxyl radicals, which could amplify peroxidation and subsequent MDA formation, and so the chain breaking antioxidant BHT is used to suppress this process during the assay (Jentzsch *et al.*, 1996; Halliwell and Gutteridge, 1999).

50μL of BHT and 250μL of phosphoric acid were added to 500μL of 0.5mg/mL oxLDL or standard and the solution vortexed. After addition of 250μL of TBA solution, the tubes were mixed by inversion and placed in a heating block at 95°C for 30 minutes with gentle shaking. The tubes were then removed and cooled on ice for 5 minutes, and centrifuged at 21,000g for 10 minutes. 20μL per sample could then be injected onto the HPLC system described above, using an Econosphere RP C-18, 4.6x50mm, 5μm column (Alltech Associates Inc. USA) and fluorescence detection with excitation at 525nm and emission at 550nm. Each run included standards of 0nmol/mL, 150nmol/mL and 300nmol/mL MDA.

PREPARATION OF PUS SAMPLES

Pus samples were obtained from patients at the DSA Unit in the Radiography department of Christchurch Hospital who gave written consent. Hospital staff transferred the pus into plastic screw top tubes, which were kept on ice for a maximum of one hour after collection until being frozen at –80°C in the laboratory.

For analysis, the pus was thawed under cold running water. The samples' preparation was carried out under aseptic conditions in a Class II biological safety cabinet. Two mL of pus was added to 2mL of nanopure water and to this mixture, 50μL each of 20mg/mL BHT and 100mg/mL EDTA were added. This solution was homogenized using a tissumizer (Tekmar Ltd, USA) for 1 minute. It was then aliquoted out for immediate

analysis by HPLC, using the pterin method above. Protein determinations were also carried out for each sample.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistica software package (Statsoft Inc., USA). Comparisons between treatments were performed using one-way analysis of variance (ANOVA). When significance was observed ($p \leq 0.05$), Tukey's multiple comparison test was done to determine if the means differed from each other by a significant margin. Significance levels are indicated in the text, or on the graphs, where the following notation is used: * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** is $p \leq 0.001$.

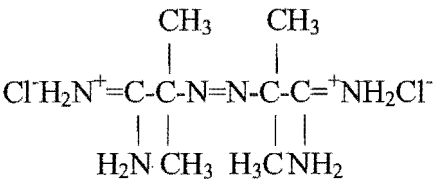
Standard deviations were calculated and are presented on the figures as error bars. The means and standard deviations are from experiments conducted in triplicate and performed at least three times.

CELL VIABILITY: MONOCYTES, AAPH AND 78NP

INTRODUCTION

This section of work concentrates on the effect of a peroxyl radical flux on two monocytic cell lines, THP-1 and U937. Peroxyl radicals are a fitting starting point for examining oxidative stress in cells, and the influence of 78NP. The radical system is likely to be simpler in its reactions than the more heterogeneous oxidised LDL, and peroxyl radicals are relevant *in vivo*, since they are generated as part of the lipid oxidation process (Gotoh *et al.*, 1993).

AAPH, 2,2'-azobis (2-amidinopropane) hydrochloride is a water-soluble, thermolabile compound, which breaks down to give carbon-centred radicals (Halliwell and Gutteridge, 1999). The radicals are generated at a known and constant rate, primarily determined by temperature, and to a minor extent by solvent and pH. The rate is directly proportional to AAPH concentration (Niki, 1990). Decomposition occurs at a first order rate constant of $k_d = 6.6 \times 10^{-5} min^{-1}$ at 37°C. A 1mM solution will therefore produce 6.6×10^{-8} mol of radicals/L/min (Esterbauer and Jürgens, 1993). The radical production reaches a plateau at 50mM (Li *et al.*, 2001).



Under suitable conditions, the molecule breaks into two carbon-centred radicals and nitrogen. In an instantaneous reaction, some of these radicals will form stable products with each other, but most will react with oxygen to give peroxyl radicals (Niki, 1990):

$$R\text{-}N{=}N\text{-}R \rightarrow 2R^{\cdot} + N_2$$
$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

AAPH has been shown to initiate a lipid peroxyl radical-mediated chain reaction in lipid micelles, erythrocyte membranes and red blood cell ghosts (Niki, 1990), and to damage DNA in cultured human T cells (Villani *et al.*, 1996). It was also responsible for the

formation of reactive protein hydroperoxides on BSA (Platt and Gieseg, 2003), U937 cells (Gieseg *et al.*, 2000A; Duggan *et al.*, 2002) and low density lipoprotein (Pearson, 2002).

AAPH causes the appearance of apoptotic features in HL-60 cells, such as DNA fragmentation, caspase-3 activation, and possibly lysosomal destabilisation (Ishisaka *et al.*, 2001). It did not cause the appearance of apoptosis in U937 cells, due to a short incubation of only six hours, but was able to inhibit the membrane Ca^{2+}-ATPase. AAPH was however said to enhance apoptosis in these cells caused by hyperthermia, in which Ca^{2+} increase and lipid peroxidation were the key events reported (Li *et al.*, 2001). The raised temperature would not only have stressed the cells, but also increased the radical flux from the AAPH.

Cell line monocytes are very useful experimental models. Cells can be obtained easily in large numbers, do not have a finite survival time, and do not display donor variability. Experiments can therefore be meaningfully compared, both within a study, and with work already published (Rodriguez *et al.*, 1994). The cell culture is also known to contain only monocytes, whereas during blood extractions, lymphocyte contamination can easily occur. Cell lines are therefore in some ways better for isolating and testing systems that may occur *in vivo* than blood-derived monocyte-macrophages (Akeson *et al.*, 1991B).

U937 is a human histiocytic lymphoma line (Château *et al.*, 1996), which was developed in 1976 by Sundström and Nilsson from the pleural fluid of a 37 year-old man with generalized lymphoma (Sundström and Nilsson, 1976; Liu and Wu, 1992). The U represents upsilon (Koren, *et al.*, 1979). It is a monoblast cell, in the early stages of monocyte development (Liu and Wu, 1992; Öberg *et al.*, 1993).

U937 cells have a mean diameter of 12.5μ. The population doubles in 95 hours. The cells have been characterised as monocytes in many tests, including positive staining with α-naphthyl acetate esterase, the presence of lysozyme in the media, which is only made and secreted by monocytes and macrophages, the existence of numerous small granules in the cytoplasm, the appearance of complement and Fc receptors on 30% of cells and the phagocytosis of test particles by 20% of cells. There was no acid phosphatase or peroxidase as in normal monocytes, consistent with the histiocytic (malignant) origin of U937 cells (Sundström and Nilsson 1976). The cells showed antibody-dependent cell-mediated

cytoxicity when activated by supernatants from human mixed cultures (Tsuchiya *et al.*, 1980).

THP-1 cells are a human monocytic leukemia cell line (Schwende *et al.*, 1996), derived from the blood of a one year-old boy with acute monocytic leukemia. The mean cell diameter is 12-14μ and the cell number doubles in 60-70 hours (Tsuchiya *et al.*, 1980).

Their characteristics are similar to the U937 line, with a few exceptions. A higher percentage of the cells are capable of phagocytosis. The cells have a different HL-A type from U937 cells, and did not show antibody-dependent cell-mediated cytoxicity (Tsuchiya *et al.*, 1980). THP-1 cells have binding sites for endothelin-1 and U937 cells do not (King *et al.*, 1997). Overall, the cell line is a more differentiated monocytic line than the U937 cells. Its ability to phagocytose, secrete TNFα and IL-1 and synthesise lipoprotein lipase and apoE support this (Banka *et al.*, 1991). Another difference is that U937 cells have around fourfold higher CD36 expression than THP-1 cells, both before and after differentiation (Nguyen-Khoa *et al.*, 1999).

Identical experiments were conducted with both the U937 and THP-1 monocyte cell types. The cells were incubated at 5 x 10^5 cells/mL with AAPH, the peroxyl radical generator, for 12 hours, and the level of cell viability or reduced thiols examined using the MTT and trypan blue assays, or the DTNB thiol assay.

All experiments exploring the effect on cell viability of a particular agent (AAPH, oxLDL or ethanol) followed the same pattern. Cells were first tested against a gradient of concentrations of the cell death mediator, and a concentration which resulted in approximately 50% viability loss was chosen for the next stage of the experiments, in which the effect of 78NP on this viability change was investigated.

Results are expressed as a percentage of the control value, to more clearly show the change in viability that resulted from the agent itself, or from 78NP, as opposed to the small viability loss due to being in a serum-free medium for a prolonged period. This presentation also allows for more meaningful comparison of results between assay methods. Experiments were repeated a minimum of three times, and a representative result is illustrated here.

RESULTS AND DISCUSSION

The viability loss in THP-1 monocytes with increasing concentrations of AAPH was concentration-dependent, levelling off by 50mM (Figure 6). This confirms previous findings (Li *et al.*, 2001). Since 10mM represented an approximately 50% viability loss, this was the concentration used in experiments involving 78NP. This experiment, and those represented by Figures 7-9, were conducted in RPMI 1640 without phenol red, and the results measured with the MTT assay.

A proportional decrease in viability with increasing amounts of AAPH was also found in U937 cells, confirming the concentration-dependent effect (Figure 7). When the two cell types were compared, the amount of viability loss at each concentration was very similar.

A difference between the THP-1 cells and U937 cells was found when the effect of increasing concentrations of 78NP on the loss of cell viability with 10mM AAPH was measured (Figures 8 and 9). THP-1 cells were unaffected by the addition of 78NP, the same loss of viability occurring whether it was present or not. But with U937 cells, viability was recovered as 78NP concentration increased, reaching significance by 200μM.

As in all experiments using 78NP, a control was included. These cells were incubated with 78NP only, at the highest concentration used. No oxidant was included. This showed that 78NP alone did not decrease the cell viability, in many cases even increasing it slightly. The increase in viability may be due to the 78NP preventing the limited amount of apoptosis that occurs in the controls (Gieseg *et al.*, 2001).

RPMI 1640 was used for these first four experiments for the same reasons that it was used with the other cell death mediators to be investigated here: it provides the cells with limited nutrition, promoting the survival of control cells and making up a more realistic, physiologically relevant surrounding. However, in this instance, the desired result of incubation with AAPH was the interaction of the cells with the peroxyl radicals themselves. Due to the possibility that constituents of the RPMI 1640 medium, such as amino acids, may also interact with the peroxyl radicals and complicate the observed effects, the remainder of the experiments in this section was conducted in Earle's Balanced Salt Solution (EBSS), a much simpler medium, consisting of glucose and salts. The effect on the cells of the lack of

nutrients may be minimal, since the incubations are only 12 hours long, as opposed to the 48 hours used in later experiments.

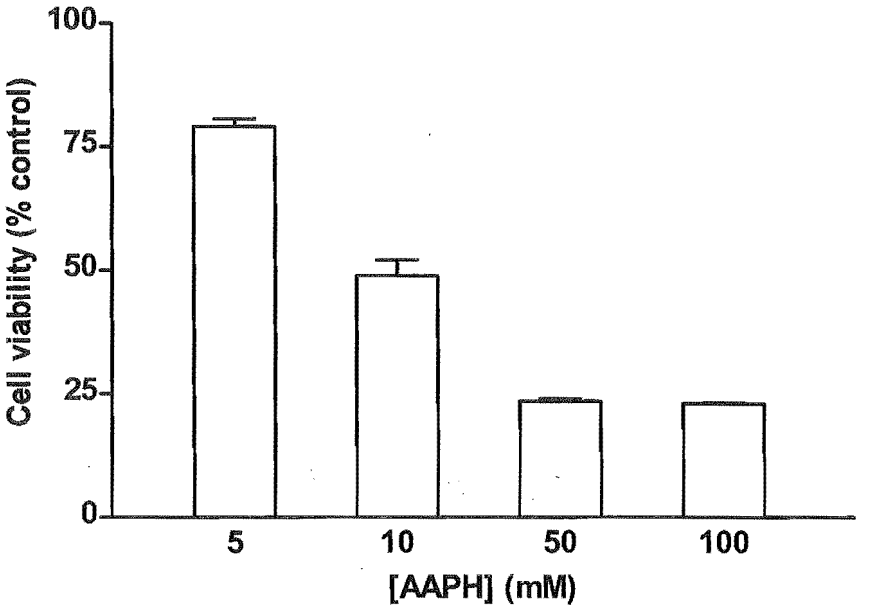


Figure 6: Effect of increasing concentrations of AAPH on THP-1 cells in RPMI 1640: MTT assay. THP-1 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in RPMI 1640, and the effect on cell viability measured using a MTT assay. The data shown is the mean ± SD of triplicates.

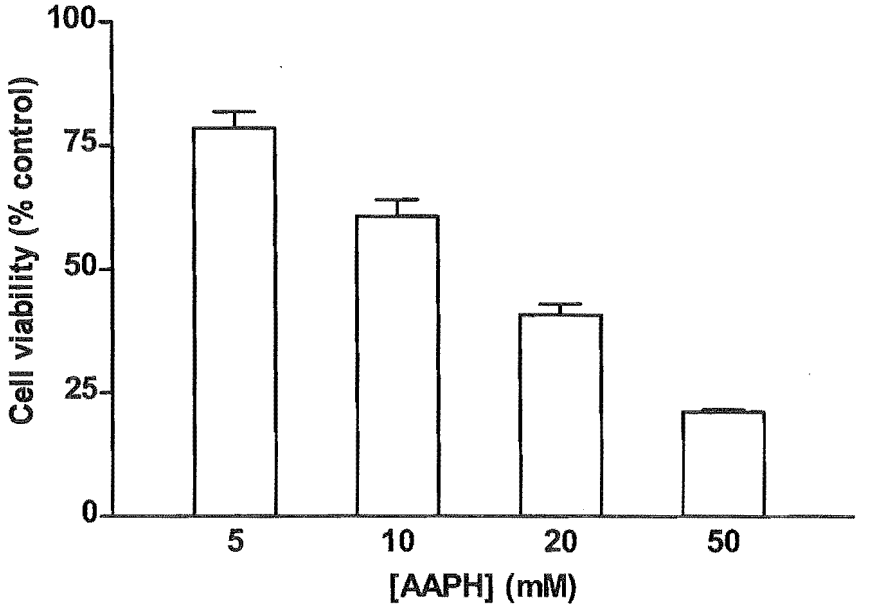


Figure 7: Effect of increasing concentrations of AAPH on U937 cells in RPMI 1640: MTT assay. U937 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in RPMI 1640, and the effect on cell viability measured using a MTT assay. The data shown is the mean ± SD of triplicates.

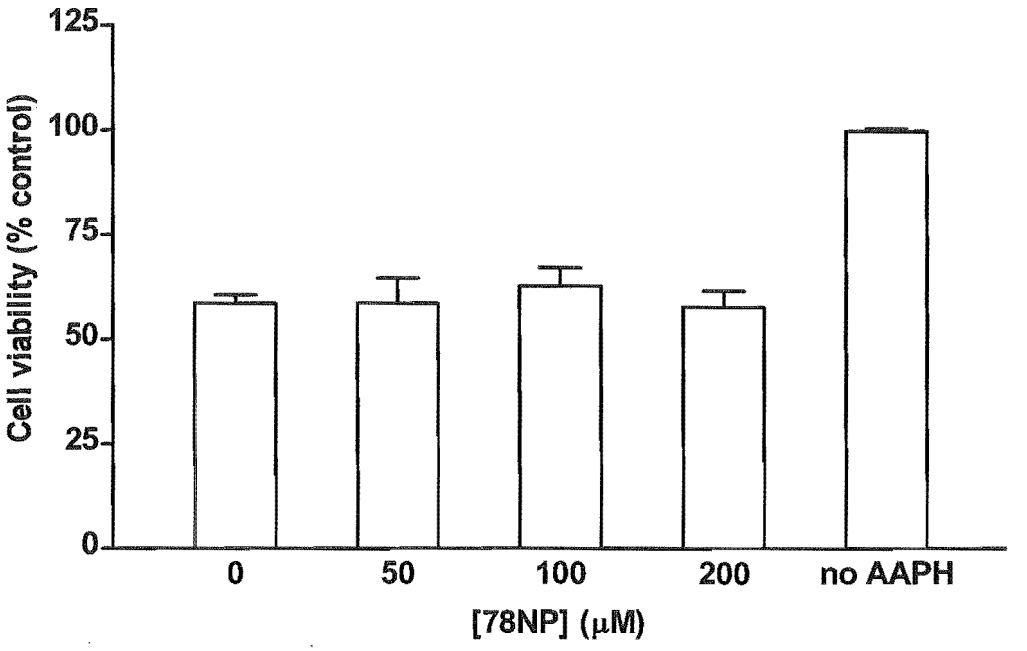


Figure 8: Effect of 78NP on AAPH-mediated decrease in cell viability in THP-1 cells in RPMI 1640: MTT assay. THP-1 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in RPMI 1640, and the results analysed by MTT assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

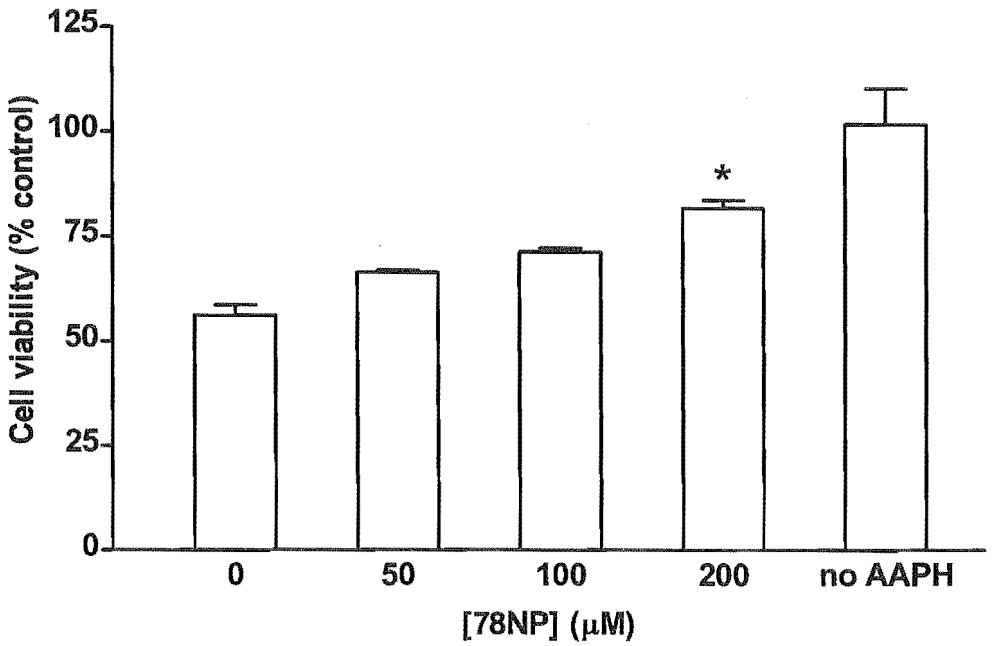


Figure 9: Effect of 78NP on AAPH-mediated decrease in cell viability in U937 cells in RPMI 1640: MTT assay. U937 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in RPMI 1640, and the results analysed by MTT assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

In spite of the difference in medium, the results of the AAPH concentration gradients in the two cell types in EBSS were very similar to those conducted in RPMI 1640 (Figures 10 -13). The actual values were very close, as were the trends: the loss in cellular viability was concentration-dependent, and the two cell types were comparable. The viability levels found by each method were broadly similar.

An effect of the medium became apparent once the 78NP was added. In contrast to the result in RPMI 1640, a small amount of protection against cell viability loss was provided to THP-1 cells by the highest concentration of 78NP used ($p \leq 0.001$), as analysed by the MTT assay (Figure 14). However, the difference between cell types was still preserved, as protection provided for U937 cells brought the viability levels back up to control levels, and significance was reached at the lower concentration of 100μM (Figure 15).

Similar results were found when the trypan blue assay was used. For THP-1 cells, the overall increase in cell viability was the same, but began at the lower 78NP concentration of 50μM ($p \leq 0.05$). The protection provided against AAPH with the U937 cells was also greater than that found with the MTT assay, reaching a significance of $p \leq 0.001$ by 50μM (Figures 16 and 17).

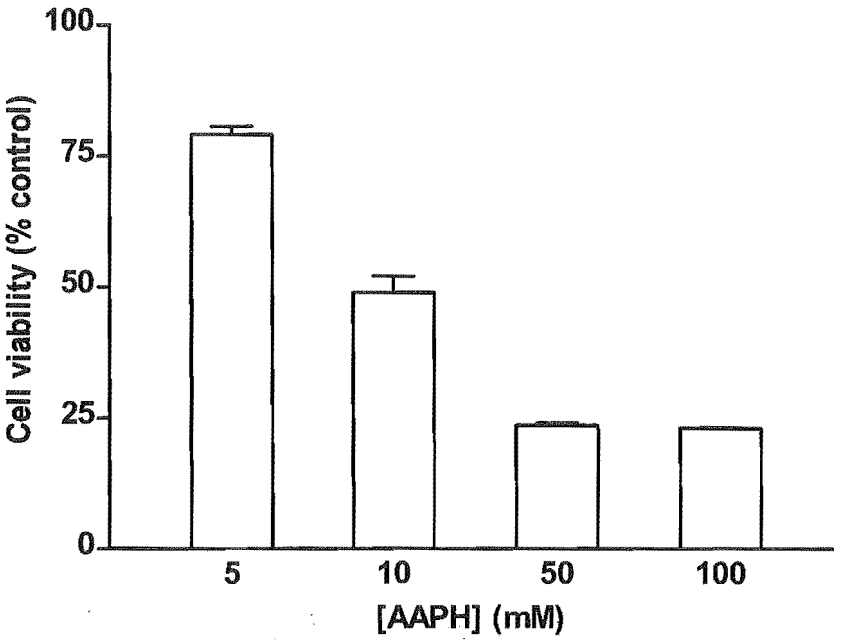


Figure 10: Effect of AAPH on THP-1 cell viability in EBSS: MTT assay.
THP-1 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in Earle's Balanced Salts Solution, and the effect on cell viability measured using a MTT assay. The data shown is the mean ± SD of triplicates.

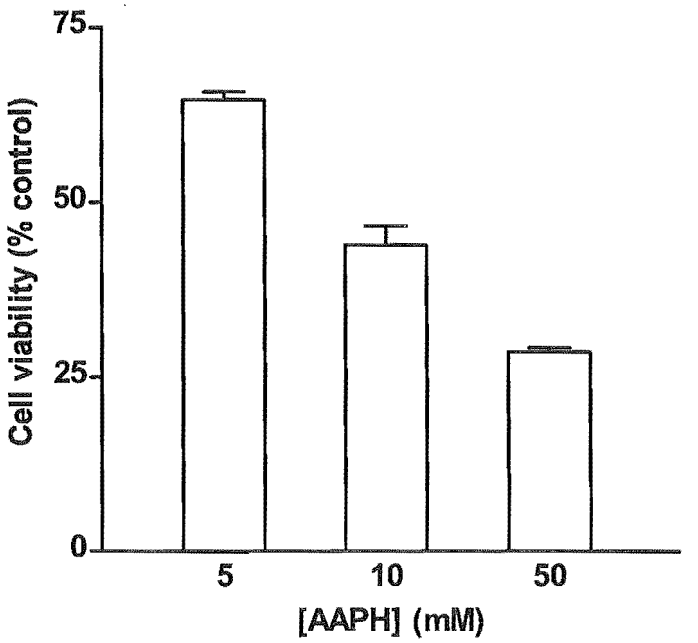


Figure 11: Effect of AAPH on U937 cell viability in EBSS: MTT assay.
U937 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in Earle's Balanced Salts Solution, and the effect on cell viability measured using a MTT assay. The data shown is the mean ± SD of triplicates.

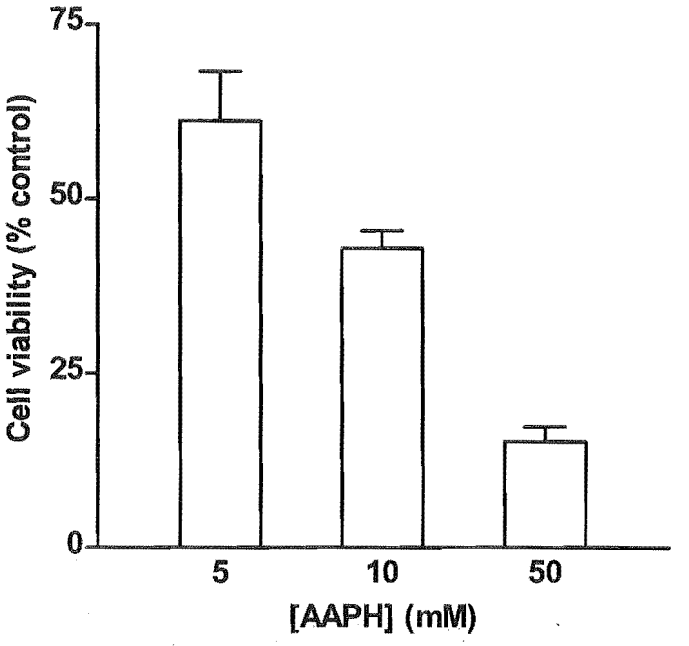


Figure 12: Effect of AAPH on cell viability of THP-1 cells in EBSS: trypan blue assay.
THP-1 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in Earle's Balanced Salts Solution, and the effect on cell viability measured using a trypan blue assay. The data shown is the mean ± SD of triplicates.

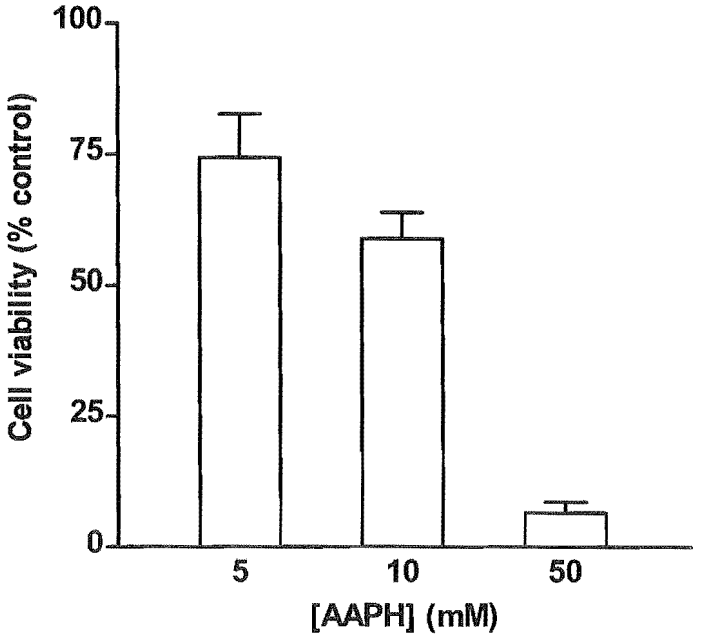


Figure 13: Effect of AAPH on cell viability of U937 cells in EBSS: trypan blue assay.
U937 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in Earle's Balanced Salts Solution, and the effect on cell viability measured using a trypan blue assay. The data shown is the mean ± SD of triplicates.

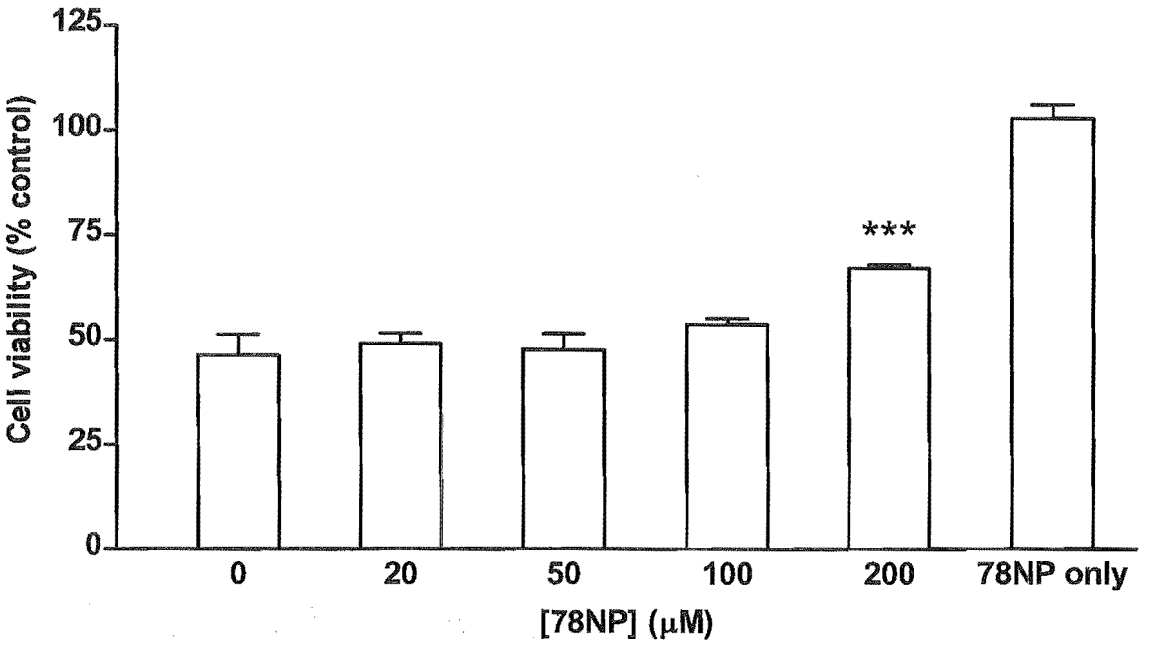


Figure 14: Effect of 78NP on AAPH-mediated decrease in cell viability in THP-1 cells in EBSS: MTT assay. THP-1 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in Earle's Balanced Salt Solution, and the results analysed by MTT assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

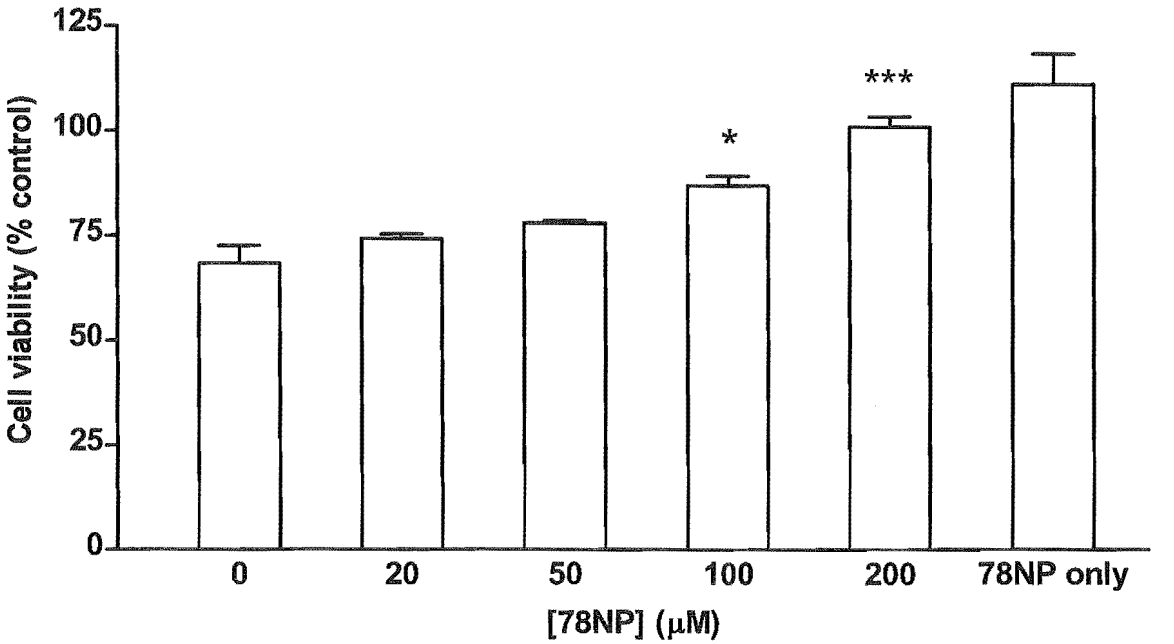


Figure 15: Effect of 78NP on AAPH-mediated decrease in cell viability in U937 cells in EBSS: MTT assay. U937 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in Earle's Balanced Salt Solution, and the results analysed by MTT assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

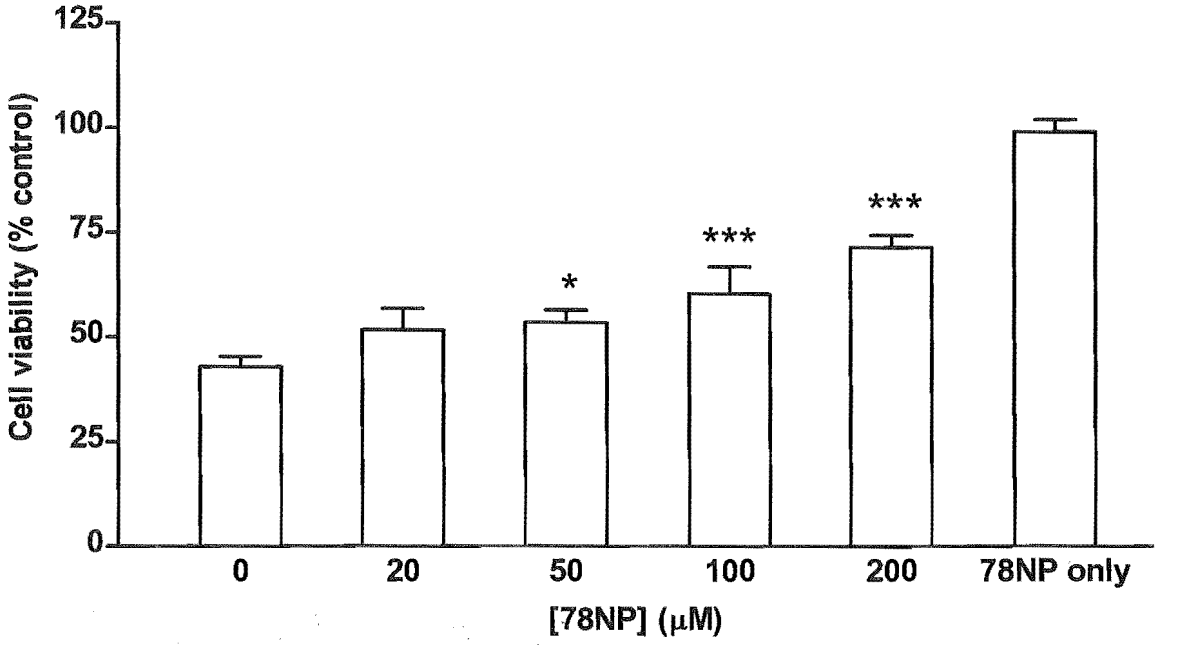


Figure 16: Effect of 78NP on AAPH-mediated decrease in cell viability in THP-1 cells in EBSS: trypan blue assay. THP-1 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in Earle's Balanced Salt Solution, and the results analysed by trypan blue assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

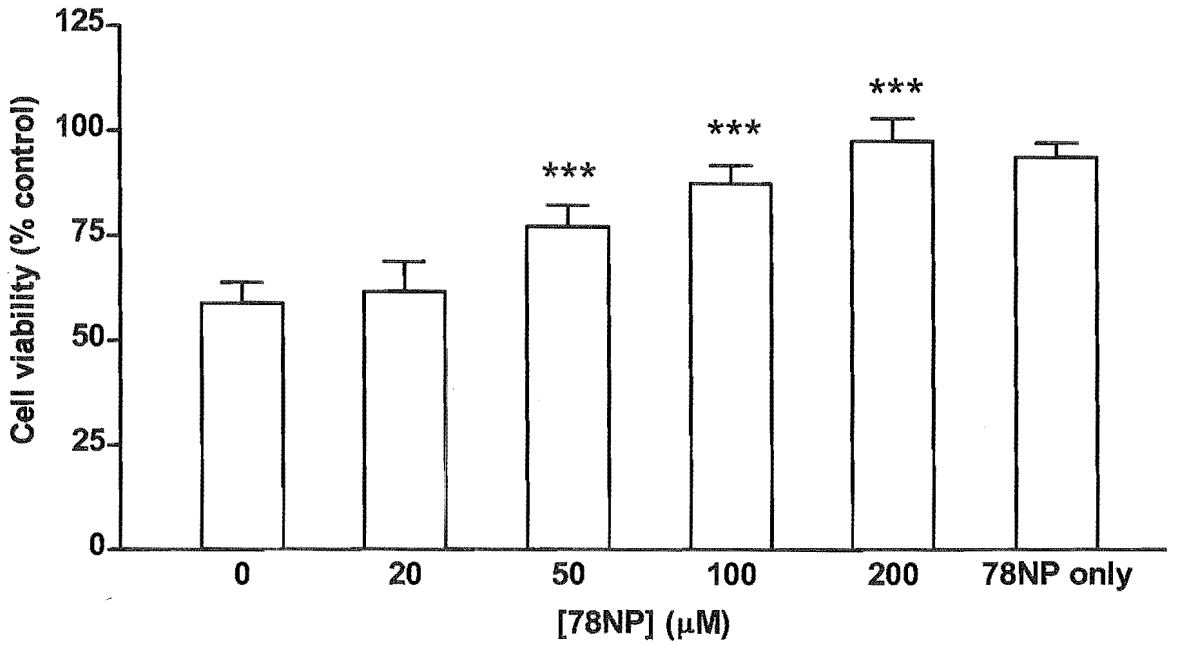


Figure 17: Effect of 78NP on AAPH-mediated decrease in cell viability in U937 cells in EBSS: trypan blue assay. U937 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in Earle's Balanced Salt Solution, and the results analysed by trypan blue assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

The DTNB assay provides a measure of the cell's total thiol redox potential, which in many cases mirrors the viability. It shows the proportion of thiol residues and thiol-containing constituents of the cell that have been oxidised as a result of cellular damage, so serving the double purpose of demonstrating oxidative effects of the so-called oxidative agents, as well as the extent of that oxidation.

The thiol loss was found to be concentration-dependent with AAPH, like the viability loss measured by the MTT and trypan blue assays and was similar for each cell type (Figures 18 and 19). At 5mM and 50mM AAPH, the percentage loss was the same as for the viability measurements. The values at 10mM AAPH, however, were much higher than their counterparts from the viability tests, showing that less thiol oxidation than viability loss occurred at this concentration. So that the comparison with these tests could be continued, 10mM AAPH was used for the experiments with 78NP in spite of this.

78NP had an effect on AAPH-induced thiol loss (Figures 20 and 21), which continues the trend of a difference between cell types. No significant difference between varying 78NP concentrations was found for the THP-1 cells, but a concentration-dependent effect, protective against thiol loss, was found with increasing 78NP concentrations in U937 cells. The thiol levels of the control were not quite attained. Significance was reached by 200μM 78NP.

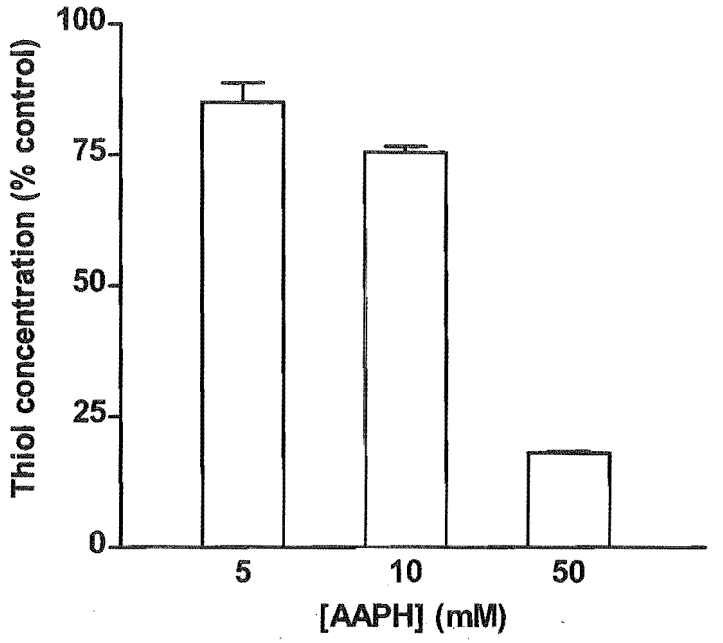


Figure 18: Effect of increasing concentrations of AAPH on reduced thiol levels in THP-1 cells in EBSS: DTNB assay. THP-1 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in Earle's Balanced Salts Solution, and the effect on reduced thiol levels measured using a DTNB assay. The data shown is the mean ± SD of triplicates.

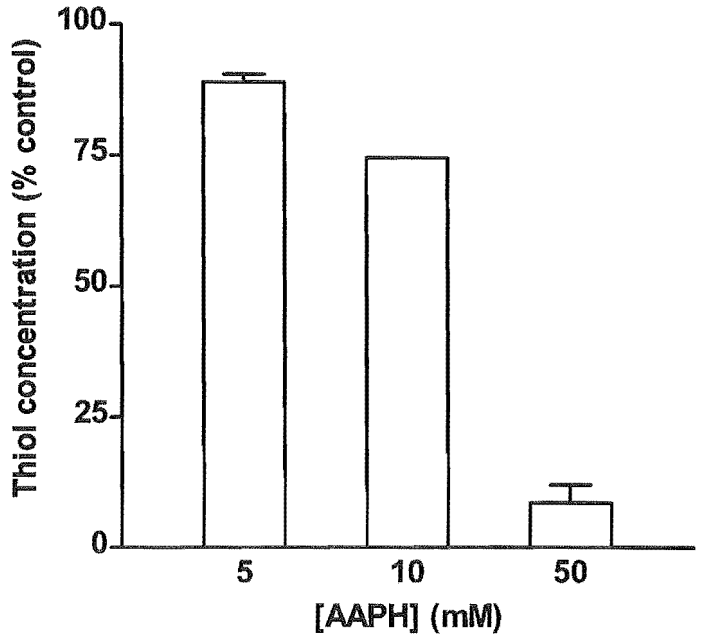


Figure 19: Effect of increasing concentrations of AAPH on reduced thiol levels in U937 cells in EBSS: DTNB assay. U937 cells at 5 x 10^5 /mL were incubated with increasing concentrations of AAPH for 12 hours in Earle's Balanced Salts Solution, and the effect on reduced thiol levels measured using a DTNB assay. The data shown is the mean ± SD of triplicates.

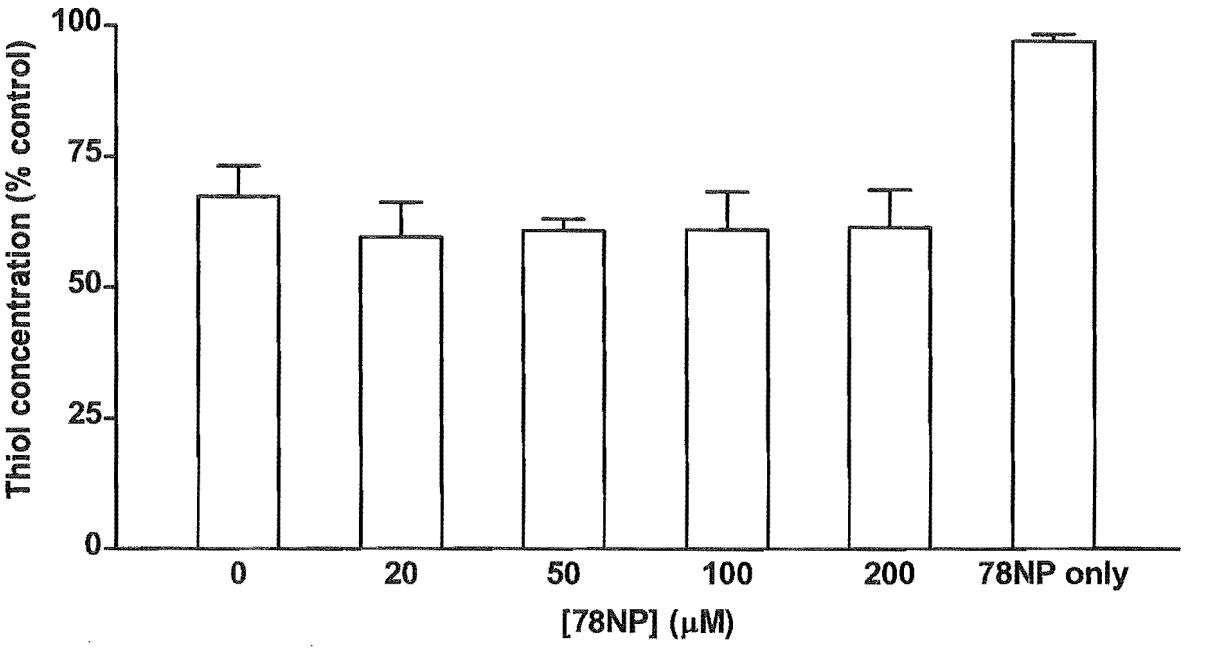


Figure 20: Effect of 78NP on oxidation of thiols caused by AAPH in THP-1 cells in EBSS: DTNB assay. THP-1 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in Earle's Balanced Salt Solution, and the results analysed by DTNB assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

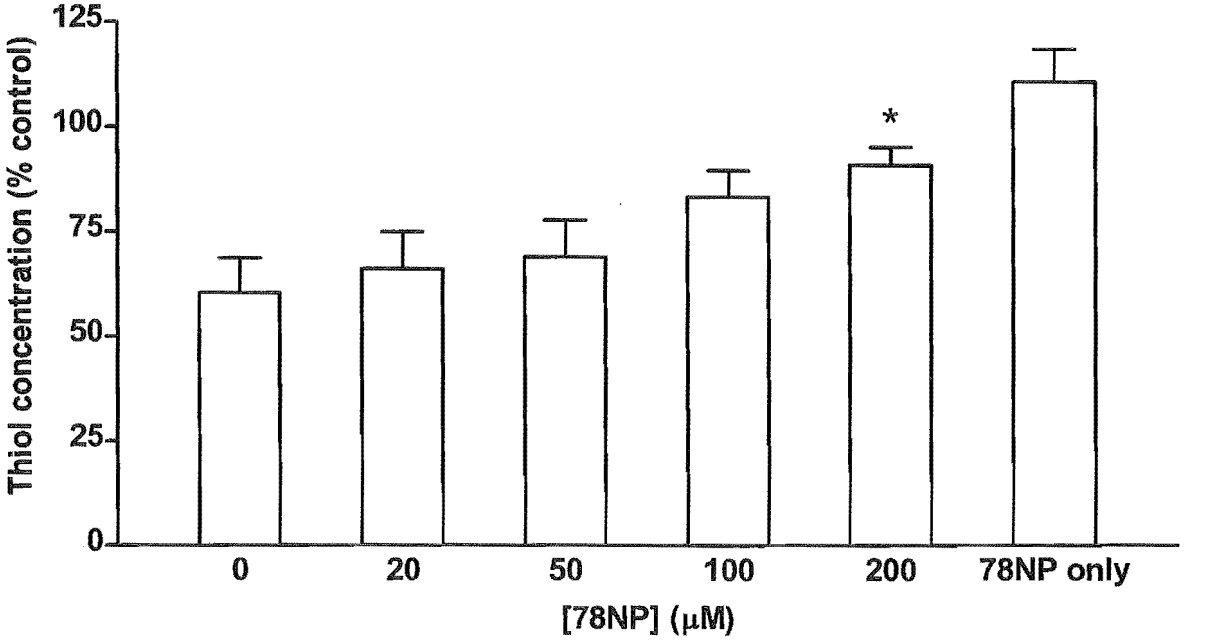


Figure 21: Effect of 78NP on oxidation of thiols caused by AAPH in U937 cells in EBSS: DTNB assay. U937 cells at 5 x 10^5 /mL were incubated with 10mM AAPH and increasing concentrations of 78NP in Earle's Balanced Salt Solution, and the results analysed by DTNB assay. A 78NP-only control is included. The data shown is the mean ± SD of triplicates.

In considering what effect 78NP might have against oxidative stress in monocytes, it would be useful to first understand what happens to AAPH once it is added to the cell solution.

The AAPH will disperse throughout the solution, forming radicals at a certain rate (determined by its concentration and the temperature). There are three possibilities for the peroxyl radicals: they may next collide with a cell and initiate lipid or protein peroxidation, or if the radical combines with a molecule that is part of the medium, oxidation products may be formed which could continue oxidation in the medium or go on to damage the cell. For example, a reaction with an amino acid could yield an amino acid hydroperoxide. Advanced glycation end products may come about through oxidation of glucose and its interaction with protein (Halliwell and Gutteridge, 1999). If glutathione was affected, thiyl radicals may result. Thirdly, if two radicals were to come together, the chain would be terminated, and a non-reactive product formed.

All of these possibilities will be found in RPMI 1640 media, but the second to a more limited extent in EBSS, which contains only salts and half the amount of glucose found in RPMI 1640. Fewer radicals would be likely to come into contact with the cell in RPMI 1640 medium, although the interaction with other reactive oxygen species would increase. It is interesting that no difference was found between the levels of viability loss in each type of medium. This implies that the secondary reactive oxygen species cause a similar level of damage to the cell as the free radicals.

Earlier work from this laboratory examining AAPH's effects on U937 cells in EBSS found that both lipid and protein peroxidation of the cells occurred, but that protein peroxidation levels appeared to have a better correlation with viability loss than did levels of lipid peroxidation (Duggan *et al.*, 2002). AAPH might then be expected to damage proteins in the cells tested here.

A positive result with the assays used itself provides information about the damage AAPH and its products are inflicting on the cell. The MTT assay examines the activity of certain enzymes important in cellular metabolic processes, such that a lowering of this activity shows a decrease in the cells' metabolism and hence available energy (Mosmann, 1983). The trypan blue assay demonstrates that cell membrane integrity has been lost, and has been used to define the end of apoptosis (Reid *et al.*, 1993C; Van den Dobbelsteen *et al.*,

1996; Hampton and Orrenius, 1997; Yuan *et al.*, 2000), and described as detecting necrosis (Escargueil-Blanc *et al.*, 1994; Meilhac *et al.*, 1999; Nonaka *et al.*, 1999; Galán *et al.*, 2001). The MTT assay point is thought to be earlier than that of the trypan blue assay, but still towards the end of the apoptotic process, since energy is required to undergo apoptosis (Carmody and Cotter, 2001).

However, the excellent correlation between the degree of cell viability loss with a certain AAPH concentration across the different assays suggests the stages of cell death being measured are very close in time. Since the trypan blue assay certainly examines a later stage, the MTT assay might be expected to do so also.

A parallel between the levels of viability loss measured by the MTT assay and a membrane integrity assay was also found in human monocyte-macrophages incubated with 1.0mg/mL oxLDL, where the lactate dehydrogenase assay was used. It was suggested that no change in MTT metabolism would be found until enough damage had been done to the mitochondria to stop them functioning, and that this was a later event (Hardwick *et al.*, 1996). In human endothelial cells with 1.0mg/mL oxLDL, trypan blue changes occurred around the same time as changes measured by the MTT assay (Escargueil-Blanc *et al.*, 1997). Therefore these assays cannot be used to prove that AAPH causes any of the characteristics of apoptosis, although necrosis or secondary necrosis is probably reached.

AAPH may cause damage to the cell either directly via radicals, or indirectly via peroxides or aldehydes formed in the medium, and this damage is intensive enough to result in primary or secondary necrosis. The damage may be focussed on protein, rather than lipid. We can also see that the process is oxidative, since a reponse to the DTNB assay is found, although we cannot tell whether the oxidation is directly due to the AAPH, or whether it is a response to other types of damage AAPH initiates. The thiol loss may be a separate event, not required for viability loss with AAPH, as the percentage of reduced thiol loss is less than the viability loss with some concentrations.

78NP might be expected to act as a scavenging, chain-breaking antioxidant in the presence of AAPH, as it can scavenge peroxyl radicals with a rate constant of 10^7 M^{-1} s^{-1}, which is close in efficiency to α-tocopherol (Oettl *et al.*, 1997). It was suggested to act in this way in experiments where it was found to lengthen the lag phase of LDL oxidation with

AAPH or Cu^{2+}. The fact that the antioxidant activity was directly proportional to 78NP concentration supported this contention. However, the same study also led to the suggestion that it was unlikely 78NP was directly scavenging peroxyl radicals in solution, as it had the same efficiency with Cu^{2+} oxidation (Gieseg *et al.*, 1995).

Both cell types in these experiments are exposed to the same radical fluxes or mixture of oxidants formed in the medium. In RPMI 1640, 78NP is unable to protect the THP-1 cells from these products at all, implying that scavenging is not the process by which it is protecting either cell type in this medium. In EBSS, where the oxidants are more likely to be peroxyl radicals, which can be scavenged, 78NP has a limited protective effect in THP-1 cells and a fairly complete one in U937 cells. So 78NP does seem to be able to act as a scavenging antioxidant to a limited extent, but its main effect in the U937 cells may occur by another mechanism. Another possibility is that 78NP or AAPH may associate with the cells in a way which limits 78NP's access to the peroxyl radicals.

The protective effect found for thiols in the U937 cells suggests this is another means by which 78NP can protect cells. 78NP could prevent reduced thiol oxidation in several different ways. It could stop them becoming oxidised in the first place, by acting as an antioxidant itself or decreasing the cell's signal to oxidise the thiols. It might also enhance their synthesis, so those oxidised could be replaced, or stop the efflux of thiols from the cell, if this is occurring. The regeneration of reduced thiols from oxidised versions is the only scenario which has been tested. Aaron Platt found in his Master's work that 78NP could not regenerate oxidised thiols on BSA (Platt, 2002). The effect of 78NP on thiols is not as great as the effect on overall cell viability in EBSS, and so thiol loss may be the only important factor, since viability can be back up to control levels without the complete recovery of all oxidised thiols.

The previously published work examining lipid and protein peroxidation in U937 cells with AAPH found that 78NP could inhibit the protein hydroperoxide formation completely at 200μM, but could not affect TBARS and lipid hydroperoxide formation (Duggan *et al.*, 2002). If 78NP is able to interact with protein, it may be in a suitable position to protect protein thiol residues. However, since it could not protect the viability or thiols of

both cell types to the same extent, and both contain protein, protection against protein peroxidation by 78NP does not always occur.

As mentioned earlier in this chapter, the THP-1 cell type is more differentiated than the U937 cells. This, or another factor, may affect the interaction of 78NP and AAPH with the cells, which has not been well studied. 78NP and the peroxyl radicals may interact with the cell membrane, or AAPH and 78NP may be taken into the cell. The THP-1 cells' ability to phagocytose in a non-specific manner, or the differing membrane composition (shown by the variation in receptor levels) might be important. The 78NP may simply be better placed in U937 cells to protect thiols and cell viability. Alternatively, the substances it affects, inside the cell or on the membrane, may initiate signalling cascades having varying effects in each cell type. This is examined in Section 2 of Chapter 4.

The difference between the two cell types may not be entirely due to differences in responses to 78NP itself. Different reactions to the oxidative stress inducers may also play a role, if 78NP is only able to be effective in one of the cases. 78NP is obviously able to have a positive effect on THP-1 cells, since not only can it protect them in very rare cases, but the incubations of cells and 78NP only, included as a control in all experiments, show that the 78NP can enhance the viability of these cells as well as it can those of the U937 type.

The disparity in differentiation of the cell types may result in each having different active metabolic pathways and responses to oxidative stresses. THP-1 cells constitutively release ceruloplasmin, whereas U937 cells do not, unless under stimulation from IFNγ. It is the U937 cells' only ferrioxidase protein, oxidising ferrous ions so that they can bind to transferrin and be removed from the cell. This may imply a higher level of iron ions within unstimulated U937 cells, and perhaps, therefore, a higher level of oxidative stress, through the Fenton reaction (Mazumder *et al.*, 1997). Although this does not seem to affect the level of cell viability loss, it may create a situation in which 78NP is more effective as an antioxidant.

SUMMARY

AAPH appears to cause THP-1 and U937 cells to respond in a similar manner. Cytotoxicity levels were not dependent on the media used or the viability assay they were measured by. Reduced thiol levels were lowered by the AAPH treatments, but not by as much as the viability levels.

78NP was always able to protect U937 cells from AAPH, in many cases bringing viability levels back up to the level of the untreated cells. It only increased viability in THP-1 cells in EBSS, and the increase was much smaller than that in U937 cells. 78NP could also decrease the loss of reduced thiols, but only in U937 cells, and did not have as great an effect as it did on the viability.

Therefore, although 78NP is an effective peroxyl radical scavenger, this study suggests that radical scavenging is unlikely to be its primary mechanism of protection in THP-1 cells, and possibly also in U937 cells. Scavenger levels are shown by the small amount of protection provided to THP-1 cells. In U937 cells, reduction of thiol loss also contributes, but since thiol levels do not return to control levels, other protective mechanisms may also play a role.

OXIDISED LDL AND 78NP: EFFECTS ON CELL VIABILITY

INTRODUCTION

This section of work examines the effect of oxLDL on the cell viability of the two monocyte cell lines used in the previous chapter, as well as the phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophage-like cell line. The protective capacity of 78NP is also tested in the more complex macrophage-like system, which may be more relevant to the *in vivo* situation of plaque development.

It has been shown previously that when THP-1 monocytic cells are incubated with phorbol esters such as PMA for periods of around seven days, they develop into cells which look, and behave, like macrophages (Akeson *et al.*, 1991A). The changes that occur during differentiation are said to make them a good model for the macrophages that are involved in atherosclerotic plaque formation (Akeson *et al.*, 1991A; Ferret *et al.*, 2000).

During PMA-induced differentiation, the THP-1 cells stop proliferating, accumulate in the G_0/G_1 phase and adhere to the plate, becoming granular and irregular in shape (Akeson *et al.*, 1991A). Membrane antigens typical of macrophages, including CD11b and CD14, are expressed (Schwende, 1996). The cells' ability to phagocytose opsonized zymosan or latex beads is also enhanced (Mouithys-Mickalad *et al.*, 2001) and receptor expression is modified. The LDL receptor is down-regulated (Auwerx *et al.*, 1989; Rodriguez *et al.*, 1994) and the oxLDL-binding scavenger receptor classes A I and II and CD68/macrosialin receptor are upregulated (Akeson, *et al.* 1991A; Geng and Hansson, 1992; Wang, 1999).

Differentiated U937 cells were not used for the work on macrophage-like cells outlined here, as differentiation of this cell type by chemical stimulus has been found to be unreliable. Some studies report phagocytosis and others do not (Koren *et al.*, 1979) and even scavenger receptor stimulation does not always occur (Via, 1989). With retinoic acid and 1,25-dihydroxy vitamin D_3 as differentiation agents, there is no cell cycle arrest (Ferret *et al.*, 2000).

Interactions between monocytes and macrophages and LDL or oxLDL are important at every stage of atherosclerotic development. During the later stages of development, oxLDL

exerts a pro-inflammatory or cytotoxic effect on cells in the plaque, which alters the plaque stability, leading to rupture. OxLDL also has a range of other effects on cells, which may complicate this situation. Those involving cytokines are likely to be less relevant here, as no other cell type is present.

Lightly oxidised or low concentrations of oxLDL, as well as native LDL, can have mitogenic effects on most cells (see Table 1). The proliferative responses can be seen in part as responses to sublethal injury, evoked to elicit repair by neighbouring cells via release of growth factors such as GM-CSF and M-CSF. This may not always be advantageous, as cells are more susceptible to toxicity during growth, especially the DNA synthesis (S phase) part of the cell cycle (Martens *et al.*, 1998; Martens *et al.*, 1999; Sakai *et al.*, 1999; Chisholm III and Chai, 2000).

Lightly oxidised LDL also has many pro-inflammatory properties, important in the early phases of atherosclerotic development, as outlined in Chapter 1, such as the recruitment of cells to the intima, the induction of growth factors, cytokines and some enzymes, such as phospholipase A_2 (type II).

More extensively oxidised LDL appears to have two major effects on atherosclerotic development: foam cell formation and stress on cells leading to cell death in foam cells and other surrounding cells. Since this study encompasses oxLDL's effects on cell viability, this type of oxLDL was used.

Lipid hydroperoxides and aldehydes can have toxic effects on cells (Table 1). It has been suggested that lipid hydroperoxides may be more toxic to cells than aldehydes (Siow *et al.*, 1999). However, when fractions of oxLDL have been compared for cytotoxicity, the neutral lipid fraction, primarily the oxysterols, is found to account for most, or all, of the toxic effect (Hughes *et al.*, 1994; Clare *et al.*, 1995; Rusinol *et al.*, 2000; Therond *et al.*, 2000; Yuan *et al.*, 2000; Garcia-Cruset *et al.*, 2001).

Table 1: Effects of LDL on cell viability

	Effect	Mechanism	Cell types studied	References
Lightly oxLDL, low concentrations of oxLDL and native LDL	Mitogenic		Human arterial smooth muscle cells, bovine vascular smooth muscle cells, lung fibroblasts, U937 monocytes, THP-1 monocytes and macrophages, human monocyte/macrophages, mouse peritoneal macrophages, T cells	Huang *et al.*, 1995; Björkerud and Björkerud, 1996; Ylä-Herttuala S., 1998; Martens *et al.*, 1999; Sakai *et al.*, 1999
Extensively oxLDL components 1.Lipid hydroperoxides	Cytotoxicity	Alkoxyl and lipid peroxyl radical formation	Human fibroblasts, human vascular smooth muscle cells	Coffey *et al.*, 1995 Siow *et al.*, 1999
2.Aldehydes	Cytotoxicity	Covalent adducts with proteins, DNA and phospholipids formed Accumulation in membranes (5-10mM) Chromosomal aberrations, point mutations, enzyme inhibition, alteration of glutathione and protein sulfhydryls, inhibition of Ca^{2+} sequestration	Human monocyte-macrophages, hepatocytes, aortic smooth muscle cells, human erythrocytes, human neutrophils, neurons	Uchida, 2000
	Signalling	ROS production, NFκB activation prevented		
3.Oxysterols	Cytotoxicity	Replacement of cholesterol in membranes Alteration of membrane structure, function and fluidity Inhibition of calmodulin HMG-CoA reductase	Human endothelial cells, human monocyte-macrophages, peripheral blood mononuclear cells, porcine aortic smooth muscle cells, Chinese hamster ovary cells.	Hughes *et al.*, 1994; Clare *et al.*, 1995; Ning *et al.*, 1996; Rusinol *et al.*, 2000; Therond *et al.*, 2000; Yuan *et al.*, 2000; Garcia-Cruset *et al.*, 2001
		Binding to microsomal anti-oestrogen binding site	MCF-7 human breast cells	

OxLDL's effects on gene expression have been explored in THP-1 cells by two 'gene surveys'. In one study, THP-1 cells were exposed to a very low concentration of highly

oxidised LDL for up to 4 days and the RNA of 10% of the genes was examined. Overall, 121 genes had increased activity and 68 were downregulated. There were no effects until after 24 hours, and most genes were affected to the highest level at day 4. Three of the products of the upregulated genes are found in foam cell-rich plaque: adipophilin, heparin-binding epidermal growth factor-like growth factor (HB-EGF) and thrombomodulin. The roles of the other genes are unknown. The genes that were downregulated were involved in promoting the anti-microbial potential of the cell, such as carbonic anhydrase, RNase A2 or CD64, or part of cell cycle progression or the synthesis of building blocks for cell division, for example thymidilate synthetase and lamin B1 (Shiffman *et al.*, 2000). This suggests that oxLDL does affect the gene expression of cells in the plaque, and that in its highly oxidised state, oxLDL may be anti-inflammatory, since genes involved in pro-inflammatory activities were downregulated.

This theory was confirmed by another study performed over only 24 hours in THP-1 monocytes also incubated with a low dose of extensively oxidised LDL. The expression of human ferritin light chain increased, meaning the cell had more iron-binding capacity. Also upregulated was the anti-inflammatory perioxisomal proliferator-activated receptor-γ (PPARγ), a member of the nuclear hormone receptor family and a key transcriptional regulator of foam cell formation and gene expression in inflammation and differentiation. 25-Hydroxycholesterol, 7-ketocholesterol, 9-HODE and 13-HODE played important roles in the upregulation; the last two are known to be ligands for PPARγ (Cader *et al.*, 1997; Jang *et al.*, 1999). Table 2 outlines the effects of extensively oxidised LDL on cell signalling pathways.

An increase in intracellular oxidative stress is likely to be involved in many of the changes in gene expression that oxLDL causes. NFκB, found in atherosclerotic lesions, is a transcription factor that controls inflammatory responses and is influenced by the cellular redox status (Draczynska-Lusiak *et al.*, 1998; Li *et al.*, 1999). Superoxide may be involved in the intracellular oxidative stress, as it has been measured in endothelial cells and U937 monocytes during oxLDL-induced apoptosis. However, preventing its production did not always delay cell death, which suggests it is not essential in signalling (Galle *et al.*, 1999B; Lizard *et al.*, 2000).

The oxLDL might also alter the cells' defenses against oxidative stress. *Tert*-butyl hydroperoxide decreased antioxidant enzyme activity over 24 hours in U937 monocytes,

including superoxide dismutase and glutathione peroxidase. OxLDL has also been shown to induce cytosolic antioxidants Cu/Zn superoxide dismutase and GSH peroxidase and mitochondrial antioxidant Mn superoxide dismutase in hypercholesterolemic rabbits and human peripheral blood monocyte/macrophages (Kinscherf *et al.*, 1997).

Other effects of oxLDL on viability that have been studied are specifically related to the apoptotic process, which is examined in Chapter 5.

Table 2: Effects of extensively oxLDL on cell signalling

	Cell types studied	Effects if studied	References
PPARγ	Monocytes and macrophages	Upregulation of CD36, cholesterol efflux Downregulation of iNOS, ROS generation, cellular oxygen consumption, metalloproteinase production, gelatinase B, scavenger R class A, inflammatory cytokines including TNFα, IL-6, IL-1β Through receptors LXRα and RXR Inhibits NFκB activation by binding to subunits	Tontonoz *et al.*, 1998; Tontonoz and Nagy, 1999; Jessup and Kritharides, 2000; Chawla *et al.*, 2001; Dominaitiene *et al.*, 2001; Fischer *et al.*, 2002; Argmann *et al.*, 2003 Chung *et al.*, 2000
JAKs (tyrosine kinases) and Stats (transcription factors)	MRC5 fibroblasts		Mazière *et al.*, 2001
Serine and threonine kinases including PKC, ERK, p38	MRC5 fibroblasts, mouse peritoneal macrophages, smooth muscle cells	p38 is necessary for PPARγ activation	Chisholm and Chai, 2000; Mazière *et al.*, 2001; Zhao *et al.*, 2002
Ca^{2+}	Polymorphonuclear leukocytes, mouse peritoneal macrophages, smooth muscle cells	PKC-dependent, part of superoxide production	Chisholm and Chai, 2000; van Tits *et al.*, 2000
NFκB Via increased intracellular oxidative stress such as superoxide Short-term exposure to oxLDL upregulates, long-term exposure downregulates	Monocytes, macrophages smooth muscle cells, endothelial cells, THP-1 cells, peripheral blood mononuclear cells	Controls pro-inflammatory responses, including TNFα, IL-1, IL-8, G-CWSF, M-CSF, GM-CSF, monocyte chemotactic protein, tissue factor, adhesion molecules	Brand *et al.*, 1997; Kinscherf *et al.*, 1997; Draczynska-Lusiak *et al.*, 1998; Galle *et al.*, 1999; Li *et al.*, 1999; Lizard *et al.*, 2000

A surprisingly small amount of work has been done on oxLDL-induced cell viability loss (usually examined as apoptosis) and effects of antioxidants. Phenolic compounds

protected endothelial, PC12 and neuronal cells from oxLDL-induced apoptosis (Draczynska-Lusiak *et al.*, 1998; Vieira *et al.*, 1998; Schroeter *et al.*, 2000; Kotamraju *et al.*, 2001).

Enzymatic antioxidants SOD and catalase inhibited apoptosis in endothelial cells (Galle *et al.*, 1999), and SOD attenuated it in mesangial cells (Sharma *et al.*, 1996).

In smooth muscle cells, γ- and α-tocopherol were effective, although α-tocopherol was more so (de Nigris *et al.*, 2000). γ-Tocopherol inhibited oxLDL-induced apoptosis in human coronary artery endothelial cells (Li *et al.*, 1999). α-Tocopherol has also been found to be protective against oxLDL in human coronary artery endothelial cells, a human epidermoid carcinoma cell line, mouse mesangial cells and vascular smooth muscle cells. The protection was only partial for the vascular smooth muscle cells and α-tocopherol acetate had a similar effect (Guyton *et al.*, 1995; Li *et al.*, 1999; Tashiro *et al.*, 1999; Li *et al.*, 2000; Masella *et al.*, 2000). Vitamin E prevented apoptosis in U937 cells incubated with 7-ketocholesterol (Lizard *et al.*, 2000).

Pretreatment with vitamin C reduced apoptosis in human vascular smooth muscle cells by 1.5mg/mL oxLDL (Siow *et al.*, 1999), and partially inhibited apoptosis in human monocyte-derived macrophages, whereas dehydroascorbic acid gave complete inhibition (Asmis and Wintergeist, 1998). Vitamin C was not effective against 7-ketocholesterol in U937 cells (Lizard *et al.*, 2000).

Results with butylated hydroxytoluene (BHT) also vary. It had no effect against oxLDL in rabbit aortic smooth muscle cells (Liu *et al.*, 1998), gave partial inhibition in vascular smooth muscle cells (Guyton *et al.*, 1995) and gave complete inhibition in human umbilical vein endothelial cells (Harada-Shiba *et al.*, 1998). BHT blocked oxLDL-induced apoptosis of mouse peritoneal macrophages (Niu *et al.*, 1996) and M1 myeloid leukemic cells transfected with p53 (Lotem *et al.*, 1996). Probucol was partially protective with vascular smooth muscle cells (Guyton *et al.*, 1995) and mouse mesangial cells (Tashiro *et al.*, 1999).

Antioxidants effective against thiol oxidation could also prevent oxLDL apoptosis. N-acetylcysteine was successful in M1 myeloid leukemic cells transfected with p53 and incubated with oxLDL (Lotem *et al.*, 1996), mouse mesangial cells with oxLDL (Tashiro *et al.*, 1999) and U937 cells with 7-ketocholesterol, as was GSH (Lizard *et al.*, 2000). N-acetylcysteine and vitamins E and C together prevented apoptosis in endothelial cells with oxLDL, by stopping caspase activation (Dimmeler *et al.*, 1997).

Cycloheximide did not protect human monocyte-macrophages against oxLDL-induced apoptosis, suggesting protein synthesis was not important. It could protect against apoptosis in macrophages with gliotoxin and thymocytes with dexamethasone (Hardwick *et al.*, 1996). NO donors and metalloporphyrins were potent inhibitors of oxLDL-mediated apoptosis in endothelial cells (Kotamraju *et al.*, 2001).

Many results, then, point towards a protective effect of antioxidants against oxLDL-induced cell viability loss, strengthening the possibility that 78NP, acting as an antioxidant, may be able to protect the cells in the same way.

The same three assays described in Chapter 3 were used to examine first the effect of different concentrations of oxLDL on the two monocytic cell lines, followed by the effect of 78NP on this cell viability change at one oxLDL concentration. A series of experiments were then undertaken to clarify the protective activity of 78NP, and to look at what may be happening to the 78NP itself as it is incubated with the cells. The influence of AAPH, oxLDL and 78NP on cell viability in THP-1 macrophage-like cells was then studied.

RESULTS AND DISCUSSION

A. MONOCYTES, OXLDL AND 78NP

1. PROPERTIES OF OXLDL

The oxLDL prepared by the procedure set out in Chapter 2 has been characterised relative to native, non-oxidised LDL. This is undertaken with two aims: to prove that the LDL is in fact oxidised, as it has characteristics typical of oxidation, and to allow its comparison with oxLDL used by other groups. The three most common sets of properties from the literature, TBARS, aggregation and relative electrophoretic mobility (REM) were used, as well as other methods commonly employed in our laboratory which provide useful information.

Table 3: Characteristics of oxLDL

Analysis	Native LDL	Oxidised LDL
TBARS	100±3nM	935±41nM
FOX assay: PrOOH	319±12nM	814±32nM
Electrophoresis: REM	9mm	29mm
Aggregation	0.001	0.005

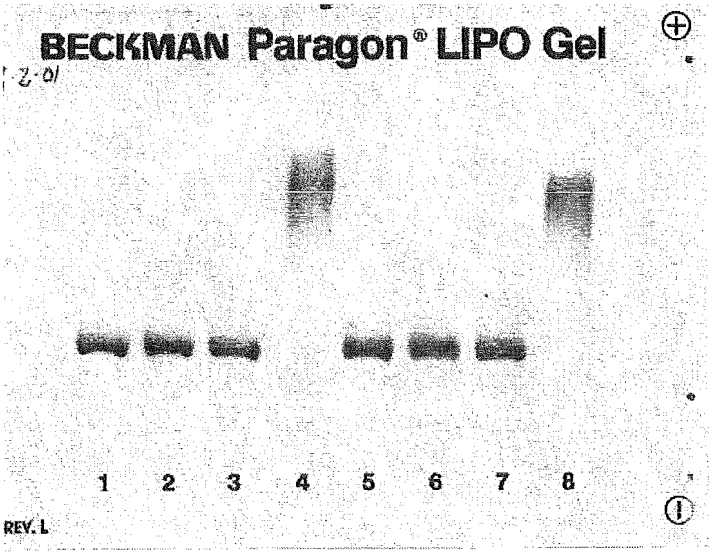


Figure 22: Lipoprotein gel of LDL. Lane 1 and 5 are non-oxidised LDL, lane 2 and 6 are LDL after incubation with 5μM Cu^{2+} for 24 hours, lane 3 and 7 are LDL after incubation with 20μM Cu^{2+} for 24 hours, and lane 4 and 8 are LDL after incubation with 50μM Cu^{2+} for 24 hours.

The TBARS HPLC lipid analysis provides an estimate of oxidative products such as lipid peroxides and aldehydes in the LDL. OxLDL was analysed at 0.5mg/mL, and it was found that there were approximately 9-fold more TBARS in the oxLDL than in native LDL (Table 3). TBARS were likely to have decayed from their peak level as oxidation progressed, since this oxLDL is extensively oxidised.

An example of reduction of TBARS over time was given by LDL oxidised at 1.0mg/mL with 100μM Cu^{2+} for 24 hours (a situation similar to the oxidation method employed here), which had TBARS increasing by 26-fold at four hours, but only 11-fold at the end of the oxidation time (Darley-Usmar, *et al.*, 1991). Less extensively oxidised LDL,

incubated with only 5μM Cu^{2+} for 20 hours, had an increase in TBARS levels of 27-fold following oxidation (Steinbrecher *et al.*, 1987).

A modified version of the FOX assay was used to measure protein hydroperoxides in LDL at 0.3mg/mL (Table 3). The low values, only 3-fold more than native LDL, reflect a decay of the peroxides over time, in a similar fashion to the TBARS levels.

The relative electrophoretic mobility of oxLDL and native LDL was compared using lipoprotein gel electrophoresis. OxLDL's mobility was 3-fold greater than that of native LDL (Table 3, Figure 22). LDL oxidised at 1.0mg/mL with 100μM Cu^{2+} for 24 hours also had a REM that tripled by the end of the oxidation period (Darley-Usmar, *et al.*, 1991). LDL oxidised by THP-1 monocytes in Hams F10 medium for 24 hours showed a 3.5-fold increase in mobility (Gieseg and Cato, 2003).

The gel shown in Figure 22 demonstrates the effect of a range of Cu^{2+} concentrations on LDL charge during the 24 hour incubation period. Lanes 1 and 5 represent non-oxidised LDL, lanes 2 and 6 represent LDL incubated with 5μM Cu^{2+}, lanes 3 and 7 with 20μM Cu^{2+} and lanes 4 and 8 with 50μM Cu^{2+}. A detectable change in REM only occurred with the highest Cu^{2+} concentration, supporting the choice of this concentration to ensure oxidation. The increase in REM is indicative of extensive oxidation, as a certain level of lipid oxidation is thought to be required before a major change in REM can occur (Fong *et al.*, 1987).

The final characterisation involved the quantification of aggregation in the LDL by a spectrophotometric measurement at 680nm. At 0.5mg/mL, the oxLDL was approximately five-fold more aggregated than the native LDL, even though it was filtered before analysis (as it was before addition to the cells), which would have removed very large aggregates (Table 1). LDL is known to aggregate during oxidation, especially in the later stages (Maor *et al.*, 1997).

There are significant differences between the oxLDL preparations of different laboratories. The concentration of the LDL when oxidised, the oxidant and its concentration, the length of the oxidation, the temperature of oxidation and transition metal or metal chelator concentration will affect the finished product (Lougheed and Steinbrecher, 1996; Ziouzenkova *et al.*, 1998). Native LDL is very heterogeneous, with different ratios of components such as PUFAs and antioxidants from each group of donors, which will also affect the properties of the oxLDL (Esterbauer *et al.*, 1988).

Oxidation of LDL by Cu^{2+} to an 'extensively oxidised' state is a good option for experimental use. The above analyses reinforce that this is the form resulting from the procedures used here. Extensively oxidised LDL shares many traits with LDL isolated from human atherosclerotic lesions, aortas with fibrous plaques and complicated lesions at autopsy. Both display similar increases in electrophoretic mobility and fluorescence at 360nm ex and 430nm em, and similar banding patterns on SDS-PAGE gels showing fragmentation of apoB. Both show an increase in TBARS levels and are recognised by antibodies to epitopes for proteins modified by MDA or 4-HNE. Both are also degraded equally by mouse peritoneal macrophages, although degradation of the atherosclerotic examples varied more between samples. The atherosclerotic samples also tended to self-aggregate more, leading to increased uptake, probably by phagocytosis (Hoff and O'Neil, 1991).

Oxidation with copper ions is an attractive oxidation method as it is simple and reproducible (Pinchuk *et al.*, 1998). This method was also chosen as it is the most common method of oxidation in the literature, increasing the ease of comparison with other studies. Another advantage is that the copper ions are easy to completely remove at the end of the oxidation period.

2. EFFECT OF OXLDL ON CELL VIABILITY IN MONOCYTES

THP-1 or U937 cells were incubated with various concentrations of oxLDL or non-oxidised LDL (as a control). The initial goal was to determine the length of incubation required to achieve around 50% cell viability loss, so that the effect of 78NP could be tested.

After 24 hours of incubation with 0.5mg/mL, 1.5mg/mL and 3.0mg/mL oxLDL, there was very little viability loss in THP-1 cells, although that which occurred was concentration-dependent (Figure 23). The 1.5mg/mL non-oxidised LDL sample gave an increase in cell viability, as it was likely to provide serum-like nutrients for the cells which are missing from the medium, and also encourages growth (Frostegård *et al.*, 1990). Only the MTT assay was used, as this was simply a test to determine incubation length.

The concentrations of oxLDL used in this study are similar to the levels of LDL found *in vivo*. The normolipidemic concentration of LDL in serum is 3.0mg/mL, 60% of the total serum cholesterol (Esterbauer *et al.*, 1992). In the artery wall LDL concentration is higher

than that of other lipoproteins, and the range is still within that found in plasma (Steinbrecher and Lougheed, 1992).

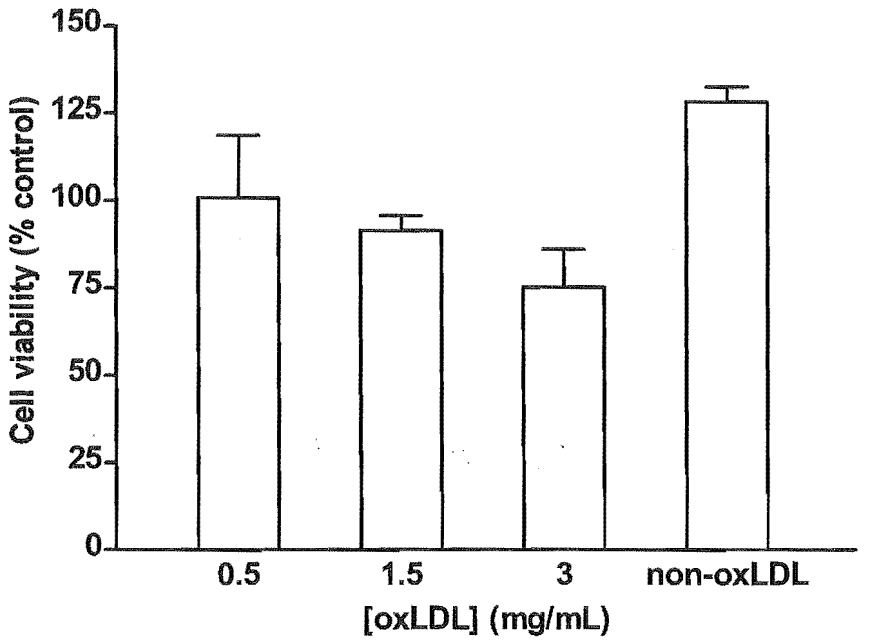


Figure 23: Effect of increasing concentrations of oxLDL on THP-1 cells after 24 hours: MTT assay. THP-1 cells at $5x10^5$ cells/mL in RPMI 1640 were incubated with varying concentrations of oxLDL for 24 hours, and the results analysed by MTT assay. A 1.5mg/mL non-oxidised LDL control was also included. The results are expressed as the mean ± SD of triplicates.

After 48 hours of incubation, 1.5mg/mL oxLDL reduced cell viability to close to 60% (Figure 24). This is the concentration of oxLDL chosen for experiments on 78NP protection. Again, the non-oxidised LDL caused no viability loss, although there was also no significant increase in viability by this timepoint. The loss in viability caused by the oxLDL was concentration-dependent in a linear fashion, and the losses measured by each assay were very similar.

As with the AAPH experiments, varying concentrations of 78NP had no significant effect on the oxLDL-mediated cell viability loss in THP-1 monocytes (Figure 25). Control cells incubated with 200μM 78NP without oxLDL did not lose viability.

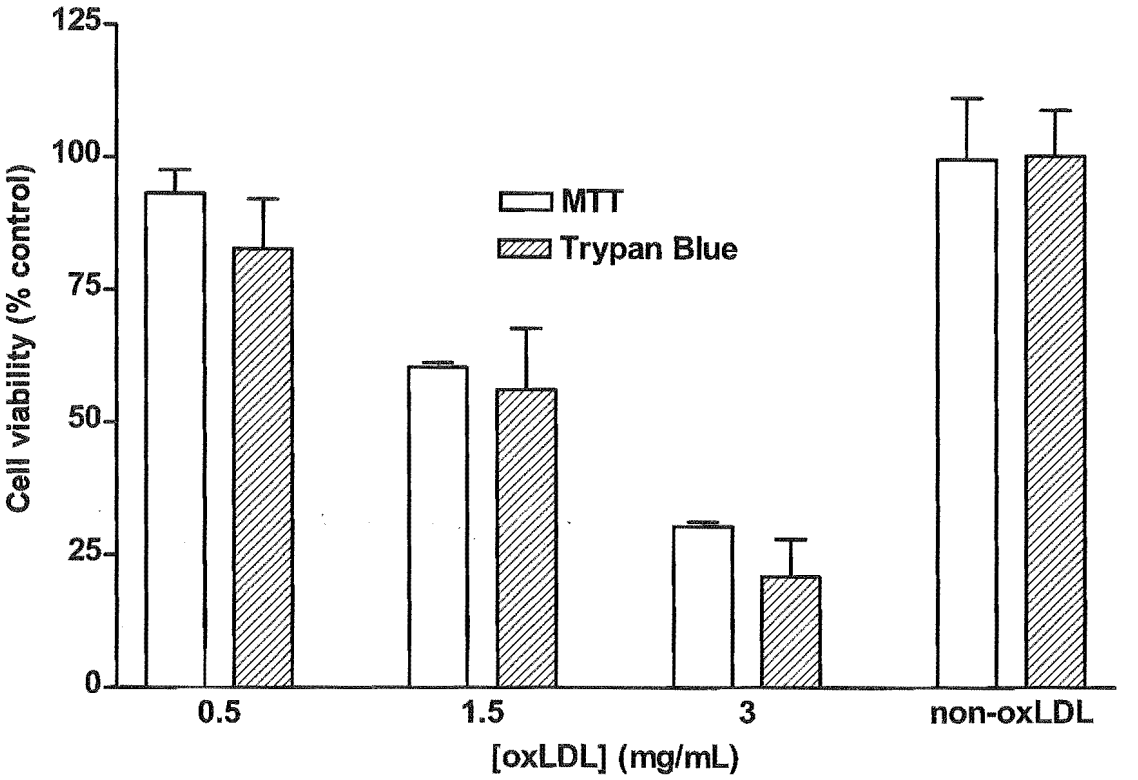


Figure 24: Effect of increasing concentrations of oxLDL on THP-1 cells over 48 hours: MTT and Trypan blue assays. THP-1 cells (5 x 10^5cells/mL) were incubated with varying concentrations of oxLDL for 48 hours in RPMI 1640, and the results analysed by MTT or trypan blue assay. A non-oxidised LDL control at 1.5mg/mL was included. The results are expressed as the mean ± SD of triplicates.

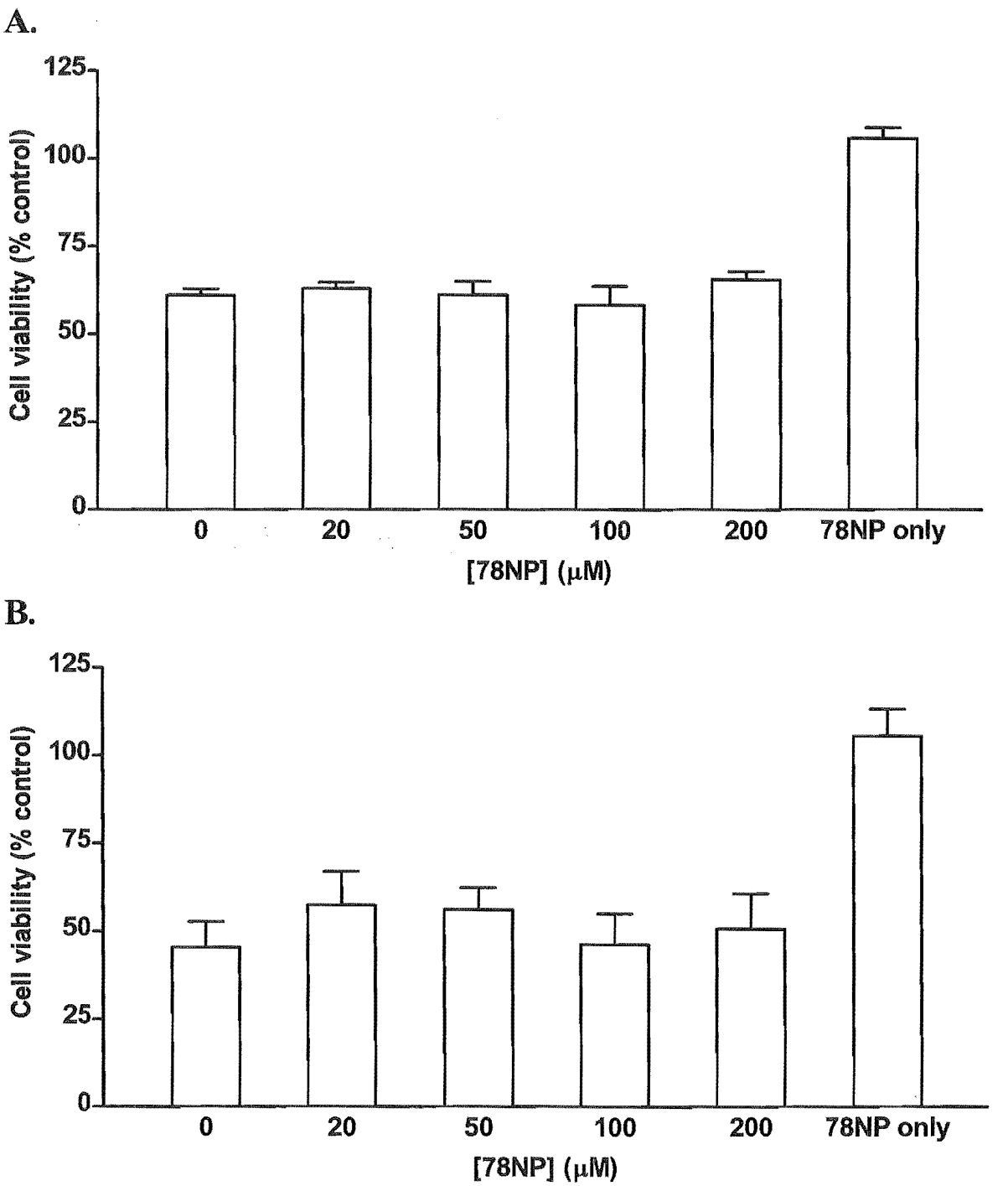


Figure 25: Effect of increasing concentrations of 78NP on 1.5mg/mL oxLDL and THP-1 cells: (A) MTT, (B) Trypan blue. THP-1 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL for 48 hours in RPMI 1640, with increasing 78NP concentrations, and the results analysed by MTT or trypan blue assay. A control with 200μM 78NP and no oxLDL was included. The results are expressed as the mean ± SD of triplicates.

The effect of oxLDL on the U937 cell type was investigated under the same experimental conditions. A 48 hour incubation with the three different oxLDL concentrations caused concentration-dependent and linear viability loss (Figure 26), similar to that observed with the THP-1 cells. The MTT and trypan blue assays yielded very similar results, and the values were also very close to those obtained for the THP-1 cells, as in the experiments with AAPH. Again, 1.5mg/mL non-oxidised LDL increased the cells' viability.

Increasing concentrations of 78NP caused an increase in cell viability in U937 cells incubated with oxLDL, which was significant at the lowest 78NP concentration tested of 10μM ($p \leq 0.01$) (Figure 27). 78NP alone at 200μM increased viability slightly.

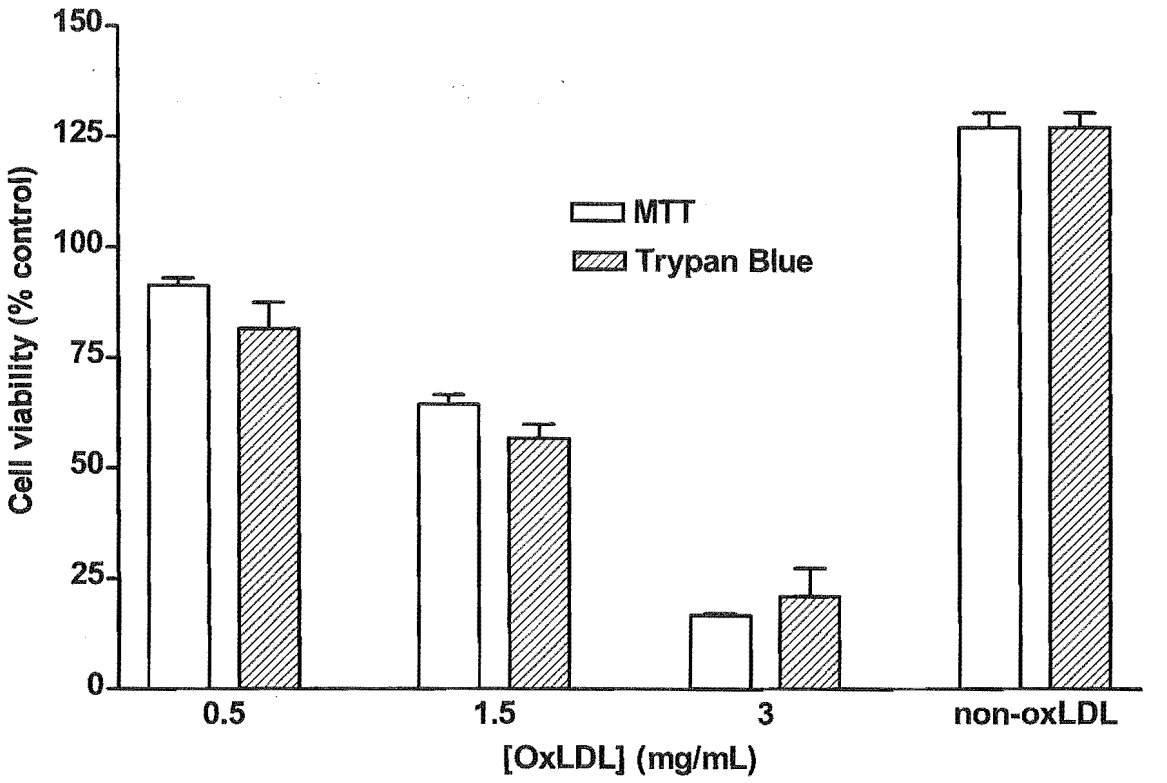
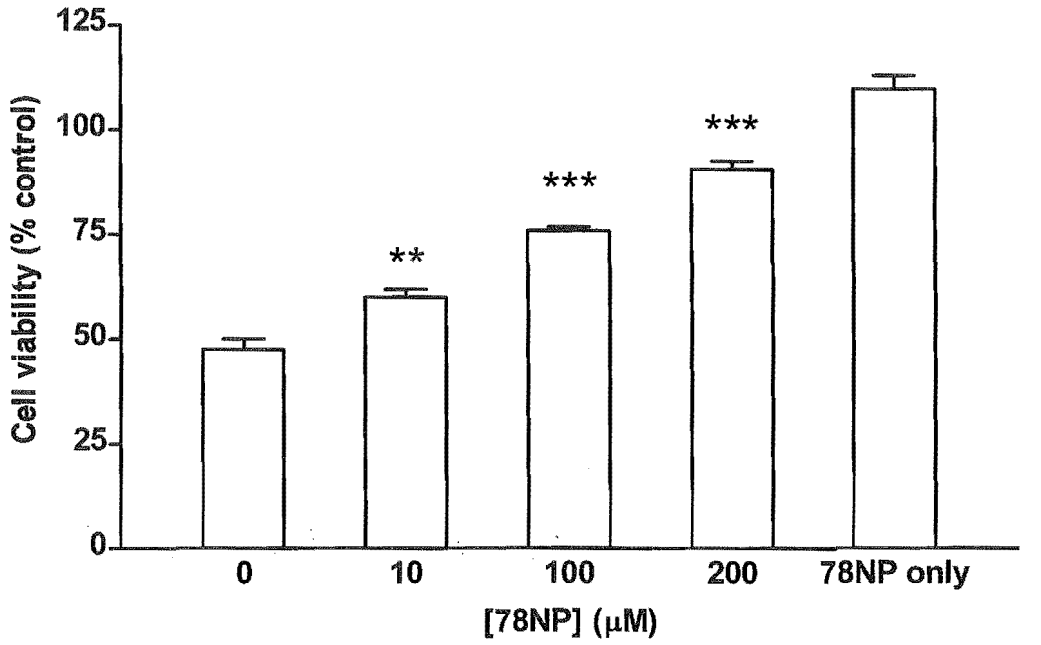


Figure 26: Effect of increasing concentrations of oxLDL on U937 cells over 48 hours: MTT and Trypan blue assays. U937 cells (5 x 10^5 cells/mL) were incubated with varying concentrations of oxLDL for 48 hours in RPMI 1640, and the results analysed by MTT and trypan blue assay. A non-oxidised LDL control at 1.5mg/mL was included. The results are expressed as the mean ± SD of triplicates.

A.



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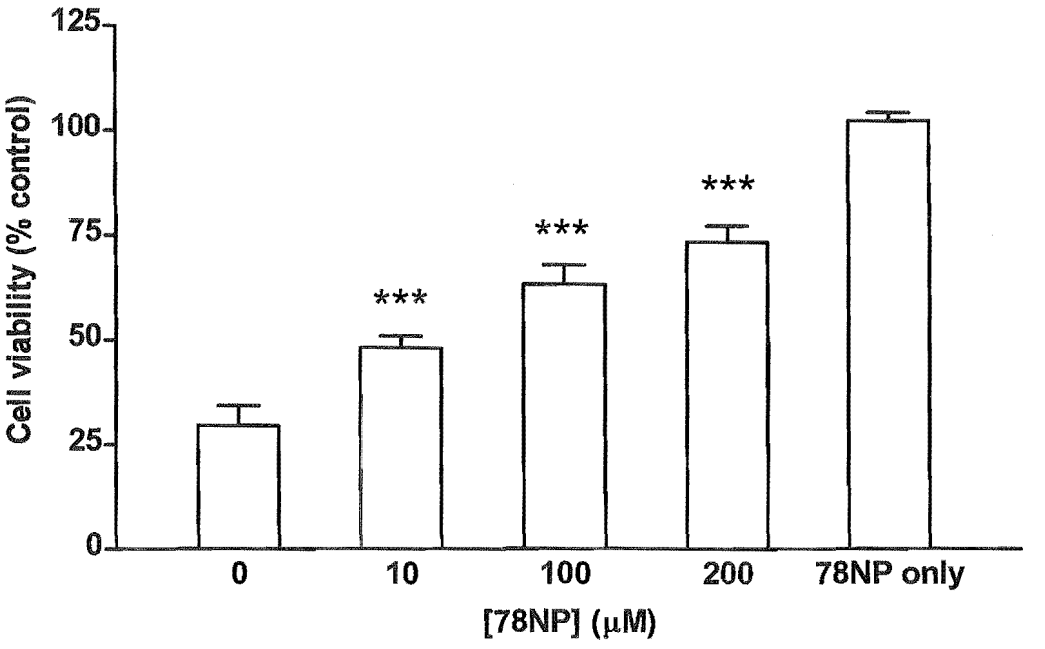


Figure 27: Effect of increasing concentrations of 78NP on 1.5mg/mL oxLDL and U937 cells: (A) MTT, (B) Trypan blue. U937 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL for 48 hours in RPMI 1640, with increasing 78NP concentrations, and the results analysed by MTT or trypan blue assay. A control with 200μM 78NP and no oxLDL was included. The results are expressed as the mean ± SD of triplicates.

Incubation of oxLDL with THP-1 cells resulted in a gradual loss of total reduced thiols, following the pattern and percentage of reduction found for viability quite closely (Figure 28). By contrast, the oxidation of thiols in U937 cells, as shown in Figure 29, was markedly different. The loss at 0.5mg/mL oxLDL was similar to the result found with THP-1 cells, but with higher concentrations the thiol levels dropped away completely. At 1.5mg/mL oxLDL, there appeared to be no reduced thiols left in U937 cells. In both cell types 1.5mg/mL non-oxidised LDL increased the level of reduced thiols slightly.

The sudden loss of reduced thiols in U937 cells was examined more closely. A greater range of oxLDL concentrations was incubated with U937 cells in Figure 30. The rapid decrease could be seen to occur between 0.5mg/mL and 1.2mg/mL oxLDL, with 0.8mg/mL acting as an intermediate point. After a critical concentration, the reduced thiols were rapidly oxidised or lost from the cell, and could not be regenerated through re-reduction or resynthesis.

The thiol loss in U937 cells over time was examined using the three original oxLDL concentrations of 0.5mg/mL, 1.5mg/mL and 3.0mg/mL (Figure 31). OxLDL at 0.5mg/mL actually caused an increase in reduced thiols before the level came back down, in this case to a little above the level of the untreated cells. With 1.5mg/mL oxLDL the initial decrease was gradual, until between 6 and 12 hours the levels dropped suddenly. Using 3.0mg/mL oxLDL the same effect was observed, but the large decrease in thiol levels occurred earlier, between 2 and 6 hours. These results support the idea of a turning-point, after which cellular thiol maintenance cannot be sustained.

The fact that these sudden thiol changes were not observed with THP-1 cells was further demonstrated in Figure 32. The alterations in reduced thiol level at 24 hours, represented here, were much the same as those at 48 hours, making it unlikely that major thiol loss had happened earlier and been partially restored.

The loss of reduced total thiols in THP-1 cells was unaffected by any 78NP concentration, whereas U937 cell thiol losses could be partially regained, although the difference was not statistically significant until 100μM 78NP (Figures 33 and 34). This mirrors the effect of 78NP on U937 cell viability (Figure 27).

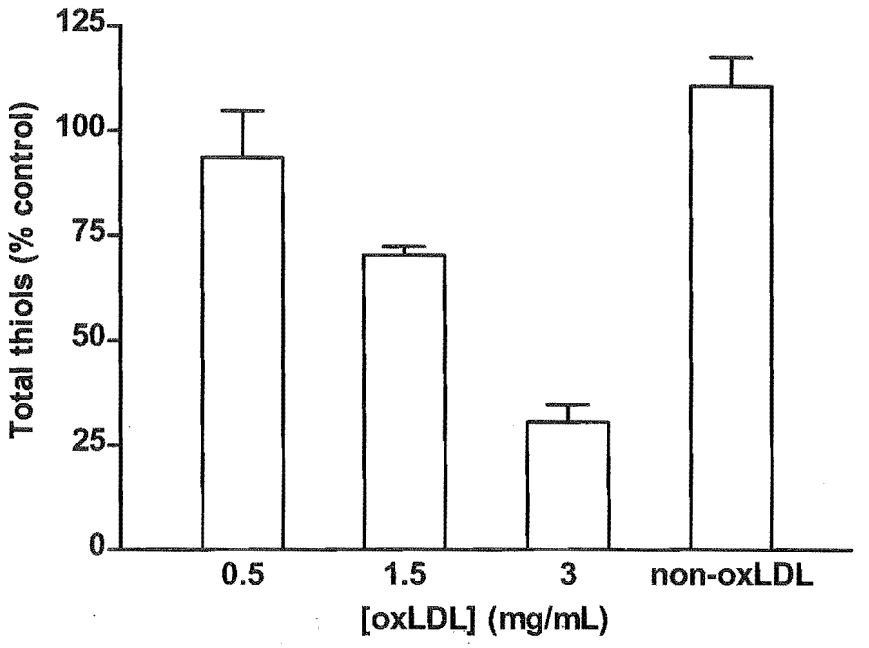


Figure 28: Effect of increasing concentrations of oxLDL on thiol content of THP-1 cells over 48 hours: DTNB assay. THP-1 cells (5 x 10^5cells/mL) were incubated with varying concentrations of oxLDL for 48 hours in RPMI 1640, and the results analysed by DTNB assay. A non-oxidised LDL control at 1.5mg/mL was included. The results are expressed as the mean ± SD of triplicates.

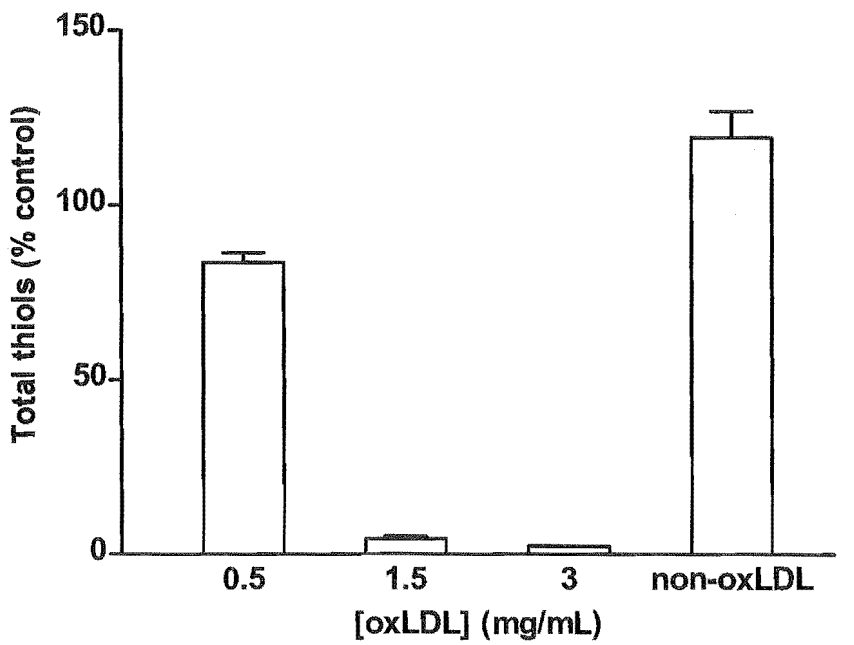


Figure 29: Effect of increasing concentrations of oxLDL on thiol content of U937 cells over 48 hours: DTNB assay. U937 cells (5 x 10^5cells/mL) were incubated with varying concentrations of oxLDL for 48 hours in RPMI 1640, and the results analysed by DTNB assay. A non-oxidised LDL control at 1.5mg/mL was included. The results are expressed as the mean ± SD of triplicates.

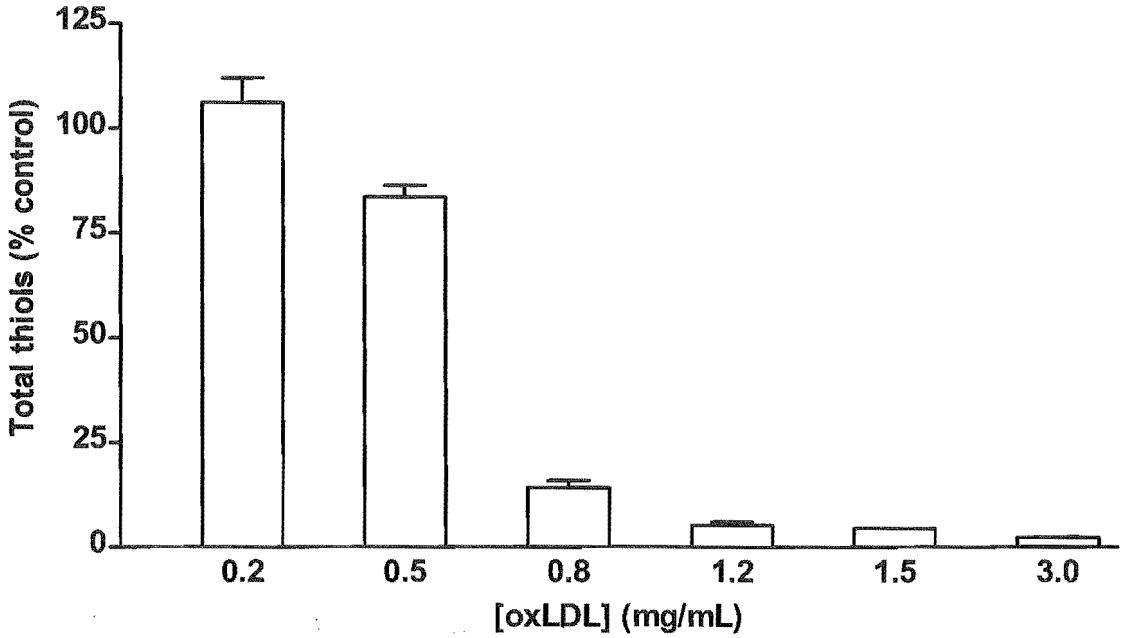


Figure 30: Effect of a wider range of concentrations of oxLDL on reduced thiol loss in U937 cells over 48 hours: DTNB assay. U937 cells (5 x 10^5cells/mL) were incubated with varying concentrations of oxLDL for 48 hours in RPMI 1640, and the thiol loss analysed by DTNB assay. The results are expressed as the mean ± SD of triplicates.

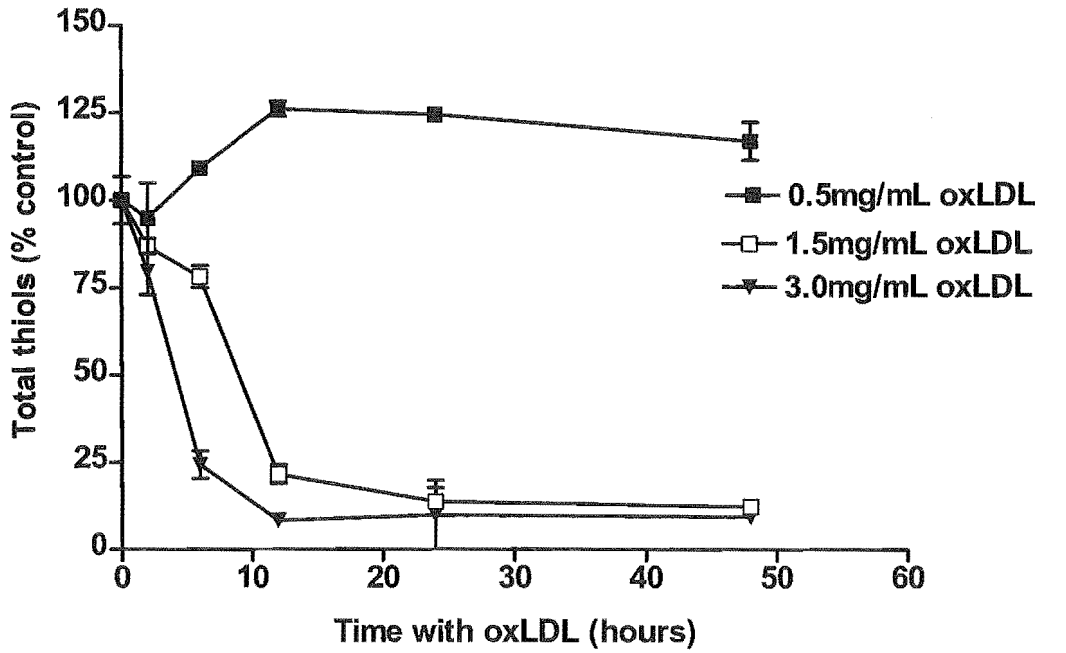


Figure 31: Effect of three oxLDL concentrations on U937 cell thiol loss over time: DTNB assay. U937 cells (5 x 10^5cells/mL) were incubated with three concentrations of oxLDL for up to 48 hours in RPMI 1640, and the thiol loss analysed by DTNB assay. The results are expressed as the mean ± SD of triplicates.

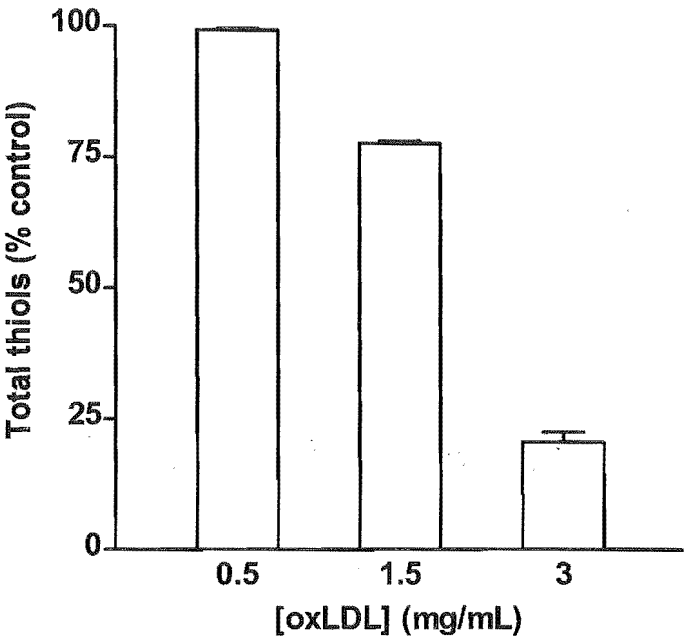


Figure 32: Effect of increasing concentrations of oxLDL on reduced thiol loss in THP-1 cells over 24 hours: DTNB assay. THP-1 cells (5 x 10^5cells/mL) were incubated with varying concentrations of oxLDL for 24 hours in RPMI 1640, and the loss of thiols analysed by DTNB assay. The results are expressed as the mean ± SD of triplicates.

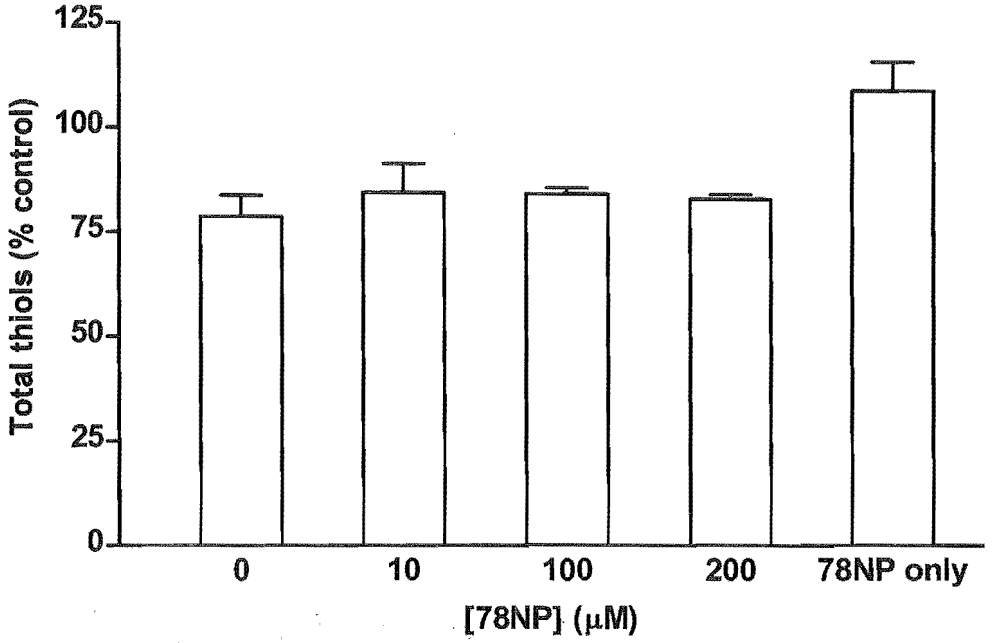


Figure 33: Effect of increasing concentrations of 78NP on reduced thiol loss in oxLDL-treated THP-1 cells: DTNB assay. THP-1 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL for 48 hours in RPMI 1640, with increasing 78NP concentrations, and the results analysed by DTNB assay. A control with 200μM 78NP and no oxLDL was included. The results are expressed as the mean ± SD of triplicates.

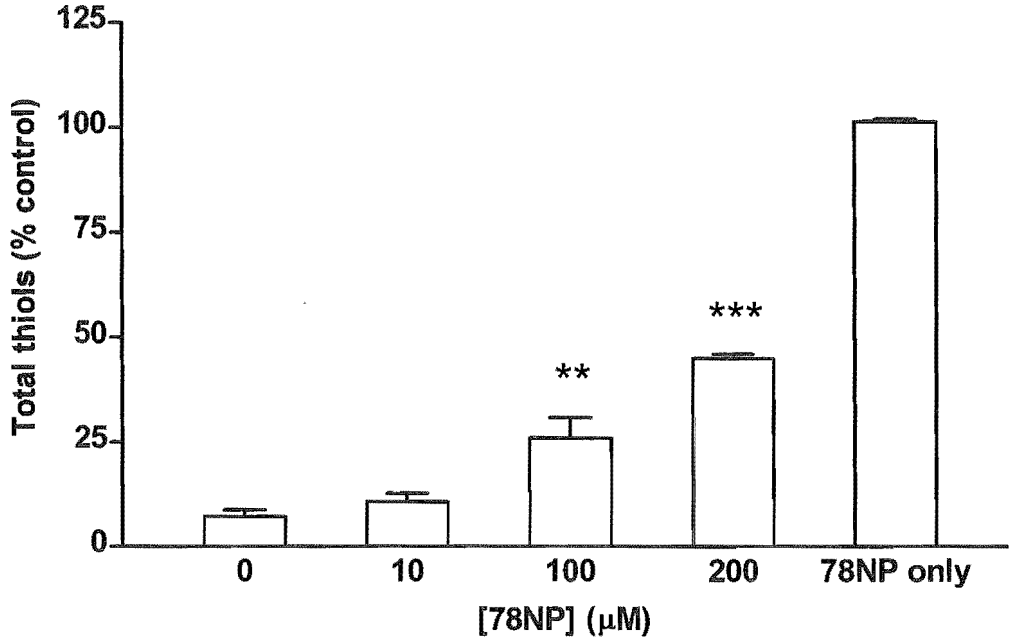


Figure 34: Effect of increasing concentrations of 78NP on reduced thiol loss in oxLDL-treated U937 cells: DTNB assay. U937 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL for 48 hours in RPMI 1640, with increasing 78NP concentrations, and the results analysed by DTNB assay. A control with 200μM 78NP and no oxLDL was included. The results are expressed as the mean ± SD of triplicates.

In order to understand how 78NP is acting to protect U937 cells, the mechanism of damage employed by oxLDL must be considered. This is investigated in more depth in Chapter 5, but nonetheless some information can be uncovered from the results found so far.

It would be beneficial to understand how the oxLDL interacts with the cells, and whether it is actually taken up by them, as occurs in foam cell formation. Most researchers agree that THP-1 and U937 cell monocytes express LDL receptors, but not scavenger receptors (Takata *et al.*, 1989; Akeson *et al.*, 1991A; Palkama, 1991; Miki *et al.*, 1996; Wang, 1999; Tanimoto *et al.*, 2001). Scavenger receptor expression is induced during differentiation of monocyte cells to macrophages with PMA (Banka *et al.*, 1991; De Kimpe *et al.*, 1998; Shimaoka *et al.*, 2000) or other phorbol esters such as TPA (Adachi *et al.*, 1993; Suematsu *et al.*, 1995).

One study found that effects of oxLDL on U937 monocytes and human peripheral blood monocytes, including chemokine receptor and p38MAPK upregulation, could be attenuated by scavenger receptor inhibitors, implying the existence of these receptors (Lei *et al.*, 2002), but this was not demonstrated or investigated further.

However, monocytes such as the cell lines used in this study might still take up the oxLDL using other receptors or mechanisms. CD36 was found to internalize oxLDL in U937 monocytes, but only to a limited extent, since the receptor was not replaced at the membrane (Pietsch *et al.*, 1996). U937 cell expression of CD36 is thought to be around four-fold higher than in THP-1 cells (Nguyen-Khoa *et al.*, 1999). A third novel form of scavenger receptor A, resulting from alternative splicing, was found in THP-1 monocytes and human monocyte-derived macrophages, but this blocked acLDL uptake (Gough *et al.*, 1998). An HDL receptor has been found in THP-1 monocytes (Webb *et al.*, 1997). Histamine and forskolin increase the expression of LOX-1 (Tanimoto *et al.*, 2001). A study using a biosensor surface with acLDL attached found at least two acLDL binding sites in U937 monocyte cells, although acLDL uptake as a result of binding was not shown by this work (Rice, *et al.*, 2002).

THP-1 monocytes are all able to phagocytose 0.8μm diameter latex particles, whereas only 20% of U937 cells do (Sundström and Nilsson, 1976; Tsuchiya *et al.*, 1980). THP-1 monocytes may ingest oxLDL by this non-specific route, although this has not been tested. Aggregated LDL may be often phagocytosed, as its uptake can be inhibited by cytochalasin B which inhibits phagocytosis (Khoo *et al.*, 1988).

Differentiation of monocytes by oxLDL during incubation could induce scavenger receptor expression. This has been found in U937 cells with 0.1mg/mL of oxLDL. Markers of differentiation such as MHCII and surface antigen Leu3 expression were enhanced, and adherence to endothelial cells was stimulated (Frostegård *et al.*, 1990; Lei *et al.*, 2002). Scavenger receptors were induced in THP-1 monocytes by oxLDL immune complexes, through binding to Fc receptors (Kiener *et al.*, 1995). The levels of oxLDL used in the work shown here is likely to be too great to cause differentiation before injury. No signs of differentiation such as an increase in size or adherence to the plate were ever observed.

Even if oxLDL were unable to be taken up by monocytes, it could still affect them in an oxidative manner by transferring some of its components into the cell membrane (Frostegård *et al.*, 1990). Aldehydes, for example, can partition from the lipid phase of the oxLDL into the cell's plasma membrane (Gotoh *et al.*, 1993).

One study with THP-1 monocytes and oxLDL found that the rate of cholesterol synthesis dropped and the rate of cholesterol esterification increased, and more cholesterol esters were stored than in human monocyte-derived macrophages. This did not happen in the presence of acLDL or native LDL (Banka *et al.*, 1991). This type of result suggests that THP-1 monocytes may well metabolise oxLDL, even though the receptor or alternative mechanism of uptake has not yet been identified.

Whatever the mechanism of interaction may be, oxLDL is certainly able to damage cells, decreasing their viability. The MTT assay shows oxLDL is able to alter the THP-1 and U937 cells' enzyme activities, metabolism and energy levels and the trypan blue assay shows an effect on cell membrane integrity, indicating that necrosis or secondary necrosis has occurred. The loss of viability was dependent on the oxLDL concentration and linear over time.

Viability loss is remarkably similar in the MTT and trypan blue assays, supporting the conclusions made in the previous chapter about the two assays measuring stages in the pathway towards cell death which are close in time. Both THP-1 and U937 cells lost viability at the same rate and had similar responses to each concentration. No disparities were found here to account for the distinct effect of 78NP on each cell type.

The fact that 78NP has no viability-improving effect in THP-1 cells with oxLDL strengthens the evidence that the oxLDL used is extensively oxidised. Lightly oxidised LDL contains many lipid hydroperoxides, which are thought to damage the cell through the creation of peroxyl and alkoxyl radicals (Coffey *et al.*, 1995). 78NP provides some protection to the THP-1 cells against peroxyl radicals, as shown in the previous chapter. The oxLDL used here will consist mainly of oxysterols and products of protein oxidation. The oxidation of apoB100 will result in conjugation to aldehydes and crosslinking causing aggregation (Esterbauer *et al.*, 1992; Meyer *et al.*, 1995). The result also suggests that the generation of free radicals as part of the cell signalling set in motion by oxLDL may not be vital to the other effects of oxLDL, since 78NP can also scavenge superoxide (Shen, 1994). Alternatively, these radicals may not be accessible to exogenously added 78NP.

The scavenging properties of 78NP may therefore not be so vital during incubation with oxLDL. The other capabilities hinted at in the AAPH studies, such as redox modulation involving thiols, may be even more important.

The first difference found in the response of THP-1 and U937 cells to an injury-provoking stimulus was the oxLDL-induced oxidation of thiol constituents. The THP-1 thiol loss was gradual and concentration-dependent, following the gradient of loss of viability obtained with the MTT and trypan blue assays. U937 cells lost their reduced thiols gradually for a short time, and then very suddenly were depleted of almost all the rest. They appeared to reach a critical point (dependent on the period of incubation for each oxLDL concentration) after which it was impossible to maintain reduced thiols. This could represent an inability to re-reduce the thiols, perhaps as the cellular environment became too oxidative. It could mean that new thiols were unable to be synthesized, or perhaps an efflux of glutathione occurred, greatly reducing the number of thiol residues in the cell.

The lowering of reduced thiols may relate to loss of activity of important enzymes or transcription factors, as well as glutathione. The p50 subunit of NFκB is thiol-sensitive, since some ROS may oxidise key cysteine 62, which is required for DNA binding. This oxidation may be part of its downregulation after a longer exposure to oxLDL (Brand *et al.*, 1997).

A loss in reduced thiol levels following oxidative stress has been reported in U937 cells treated with *tert*-butylhydroperoxide (tBOOH). tBOOH oxidises glutathione, causing an

increase in GSSG intra- and extracellularly after two hours of incubation (Nardini *et al.*, 1998). GSH was extruded before membrane leakage in U937 cells with puromycin (Ghibelli *et al.*, 1998). In U937 cells with 7-ketocholesterol, down-regulation of antioxidants including glutathione and a decline in transcription levels of catalase, superoxide dismutase and thioredoxin were found (Lizard *et al.*, 1998).

The thiol changes show that, in spite of the very similar overall viability losses, oxLDL does act on each cell type differently. The oxLDL had a minor oxidative effect in THP-1 cells, lowering reduced thiols in parallel with viability, as did AAPH in both cell types. Thiol loss might not be the defining event in viability loss in THP-1 cells with oxLDL or AAPH. Native LDL increased thiol levels, as part of its improvement of the cell's overall viability, and 78NP alone had a similar effect. This suggests that reduced thiols do contribute to viability in THP-1 cells in some way.

Also noteworthy is the increase in reduced thiols found with the lower oxLDL concentration. This has been reported before in a range of cell types. Both THP-1 monocytes and macrophage-like cells incubated with 0.5mg/mL extremely oxidised LDL experienced an initial small decrease in glutathione levels, and an elevation by 24 hours. It was found that if cells were pretreated with a non-toxic oxLDL dose, the increase in glutathione would protect the cells against a subsequent toxic dose of 2.0mg/mL (Gotoh *et al.*, 1993). In THP-1 cells incubated with 0.5mg/mL oxLDL for 24 hours, glutathione levels dipped in the first three hours, then increased, to a greater level with more oxidised LDL. No loss of viability was found (Darley-Usmar, *et al.*, 1991).

Low concentrations of lightly oxidised LDL at first decreased GSH in human vascular endothelial cells, but the levels were doubled by 24 hours, in a dose dependent fashion between 0.125mg/mL and 0.5mg/mL oxLDL. The effect was not found with native or acetylated LDL. OxLDL stimulated the transcription of γGCS via increases in AP-1 DNA binding activity mediated by reactive oxygen species (Cho *et al.*, 1999).

In U937 cells, oxLDL's oxidative effects were much greater than its overall effect on viability. Reduced thiols were completely abolished, suggesting that the oxLDL may be well placed to oxidise them in this cell type, or may set in motion a chain of events that causes this oxidation, which it does not in THP-1 cells. Since the complete loss of thiols did not occur

with AAPH in U937 cells, it not an obligatory part of their response to oxidative stress; rather it is something more particular for the oxLDL.

Different fractions of oxLDL have varying effects on glutathione loss in human endothelial cells. The depletion of reduced thiols did not necessarily correlate with the cytotoxic effects. 7-Ketocholesterol fractions caused a greater percentage of viability loss than glutathione loss, whereas aldehydes depleted 100% of the glutathione and caused less viability loss. Oxysterols cannot be conjugated by GSH, which may play a part in the difference (Therond *et al.*, 2000).

Possibly the difference in thiols in the cell types may relate to a difference in signalling pathways set in motion by oxLDL. If U937 cells do not express transcriptional regulator PPARγ in response to oxLDL, as suggested (Inoue *et al.*, 2001), and THP-1 cells do, a longer incubation with oxLDL would not have the anti-inflammatory effects in U937 cells that would be found in THP-1 cells. ROS generation might not be reduced, which would impact directly on reduced thiol levels.

The difference between the effects of AAPH and oxLDL on thiols in U937 cells may reflect different degrees of influence over intracellular oxidative balance. U937 cells were found in one study to have a lower level of glutathione expression than THP-1 cells. They also had much less thioredoxin and thioredoxin reductase, almost no thioredoxin at all. They had slightly higher levels of MnSOD (Ferret *et al.*, 2000). U937 cells' antioxidant defenses may be weaker overall, or less oriented towards the type of oxidative stress produced by oxLDL. If the U937 cells have higher levels of superoxide dismutase, they may be better able to cope with a radical flux than stress caused by non-radical reactive oxygen species.

The more spectacular drop in thiols in U937 cells may well account for at least some of 78NP's protective ability in this cell type, and lack of it in THP-1 cells, as was the case with AAPH. U937 cells may be more sensitive to an oxidative environment, as suggested by the loss of their thiols. Loss of thiols would then certainly exacerbate the oxidative effect of the oxLDL. The increased oxidative stress may mean that 78NP can therefore play a role in reviving the cell, by helping to create a more reducing environment. It may lower or slow oxidation of the thiols.

Increase in reduced thiols has been reported to improve U937 cell viability in a range of circumstances. Addition of GSH or its precursor N-acetylcysteine halved loss of viability

with 7-ketocholesterol. GSH and N-acetylcysteine could also impair etoposide but not cycloheximide viability loss (Lizard *et al.*, 1998). Thiol loss was suppressed by N-acetylcysteine and enhanced by BSO in U937 cells with the 2-oxoaldehydes methylglyoxal and 3-deoxyglucosone and this was reflected in viability (Okado *et al.*, 1996). U937 cells lost 50% of their reduced glutathione with tBOOH by 2 hours, and the addition of caffeic acid halved this loss (Nardini *et al.*, 1998).

Low micromolar concentrations of neopterin were found to activate NFκB, and both 78NP and neopterin enhanced cGMP effects and transiently increased intracellular Ca^{2+} levels (Barak M. and Gruener N., 1991; Woell *et al.*, 1993; Schobersberger *et al.*, 1996; Murr *et al.*, 1999). Since longer incubations with oxLDL downregulate NFκB, and oxLDL also alters Ca^{2+} levels, the protection by 78NP may involve the reduction of these oxLDL effects. However, since 78NP can only affect cell viability loss with oxLDL in U937 cells, whichever pathways 78NP alters to improve viability must only be important for the effect of oxLDL in the U937 cell type. Other pathways may take on a greater significance in the absence of PPARγ in these cells.

If there is a difference in cell uptake, the 78NP may be in a position where it can undertake this protective role with the thiols. For example, with Jurkat cells and anti-Fas antibody, it was found that an external antioxidant could lessen the impact of glutathione loss, and hence delay, although not prevent, apoptosis (Van den Dobbelsteen *et al.*, 1996).

This may be not the only way in which 78NP acts to inhibit oxLDL-mediated cell death. The next set of experiments aims to clarify its possible roles and the ways it carries them out.

B. CHARACTERISING THE PROTECTIVE EFFECT OF 78NP

1. THE STABILITY OF 78NP OVER THE TIME OF INCUBATION

To determine the stability of 78NP during an experiment, 200μM 78NP was incubated with THP-1 monocytes or RPMI 1640 for up to 72 hours, in a mimic of an experimental situation. The concentrations of three compounds, 78NP, neopterin and 7,8-dihydroxanthopterin (78XP), were examined by HPLC.

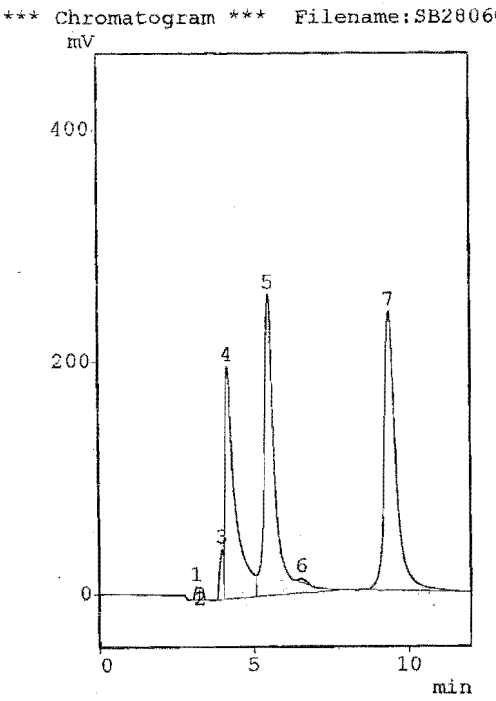
The major oxidation product was found to be 78XP (Figure 35). The sample chromatogram shows 78NP after incubation with cells for 12 hours. In the ECD chromatogram, peak 5 represents 78NP and peak 7 is 78XP. As time went on, peak 5 shrunk, and peak 7 grew from an imperceptible size to become very large. On the fluorescence chromatogram, peak 7 is 78NP/neopterin, and 78XP does not show up. The 78NP/neopterin peak diminished over time.

The results from the ECD detector showed that the 78NP concentration slowly decreased over the 72 hours, until it had all been converted to 78XP, which steadily increased (Figure 36). The 78NP incubated with the cells degraded at a slower rate, with a concurrently slower pace of 78XP formation, suggesting the cells were stabilising the 78NP. Effluxed thiols, reductants or antioxidants may have been responsible for this, and the cells' own production of a small amount of 78NP may also have contributed.

Since the fluorometric peaks of neopterin and 78NP are separated by only 0.2 minutes, they cannot be distinguished by this analysis. Examining the size of the peak, it can be seen that some neopterin must have been present, since otherwise much more than the original 200μM 78NP was detected (Figure 37). Since from the standards we know that neopterin is around threefold more fluorescent than 78NP, that amount can be calculated. The levels are small, but present from the start.

An interesting difference between the samples with and without cells was also found here. When cells were included, the concentration of neopterin rose slightly over time, suggesting the cells may be producing a small quantity, or else another metabolite they release may encourage some oxidation of 78NP towards neopterin. The cells may also stabilise the neopterin, since without the cells, the neopterin component appeared to break down, decreasing in concentration over time.

This data showed that cells, at least without the presence of an oxidant, appear to stabilise 78NP rather than increase its oxidation. 78NP did not appear to be being taken up by the cells in great amounts, as close to the full 200μM of 78NP or 78XP was always detected without lysis of the cells.

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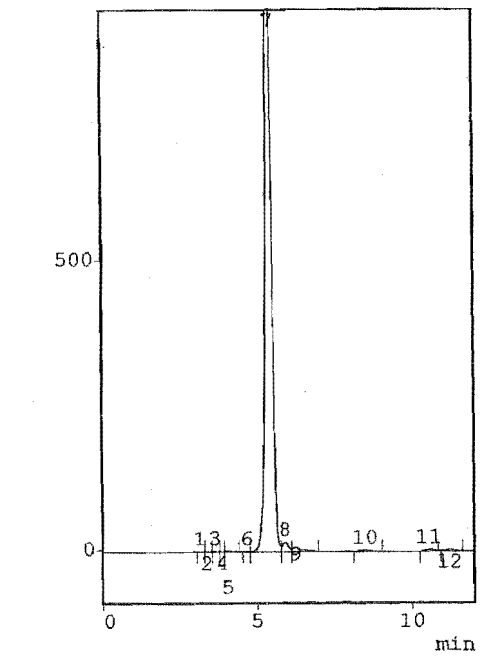
Vial #          : 4
Sample          : cells + 78NP
Inj. Volume     : 10
Detector        : Other
Operator        : Sarah
Method Name     : NP2C_ECD.MET

*** Peak Report ***
```

PKNO	TIME	AREA	HEIGHT
1	3.134	84486	10672
2	3.263	90250	10584
3	3.958	365981	42431
4	4.148	4532961	199328
5	5.459	6132137	259051
6	6.527	54243	3120
7	9.364	6337736	239679
		17597794	764865

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Detector        : RF-10AXL

*** Peak Report ***
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PKNO	TIME	AREA	HEIGHT
1	3.142	1920	152
2	3.375	3569	303
3	3.629	5714	536
4	3.875	3627	415
5	4.047	12519	911
6	4.658	1896	214
7	5.425	15586498	1003530
8	5.896	212390	15323
9	6.242	127862	7391
10	8.478	39078	1543
11	10.531	36349	2338
12	11.196	41048	2236
		16072470	1034892

Figure 35: Sample chromatogram from HPLC analysis of the stability of 78NP over 72 hours. This chromatogram is from a sample of 78NP incubated with cells, and analysed at 12 hours using the pterin method with both the ECD and fluorescence detectors. The top chromatogram shows the ECD results. Peak 5 represents 78NP and peak 7 78XP. The lower chromatogram is the fluorescence detector trace, the single peak shows 78NP and neopterin.

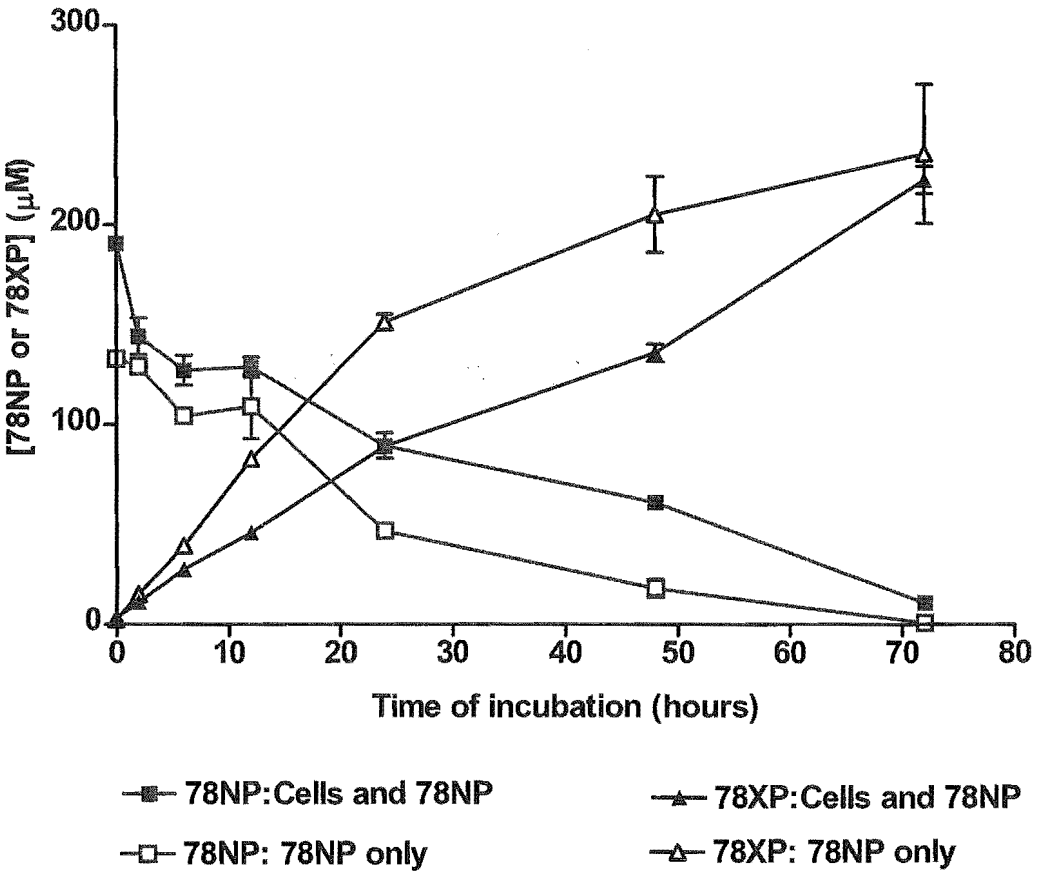

Figure 36: The stability of 200μM 78NP over 72 hours with and without cells.
200μM 78NP was incubated in RPMI 1640 with or without THP-1 cells (5 x 10^5cells/mL) at 37°C for 72 hours. The concentration of 78NP and 78XP was measured at each timepoint using the pterin HPLC method without oxidation with acidic iodide solution, using the ECD detector. 78XP concentration was calculated using a fluorometric ratio of 3:1 with 78NP, determined earlier by HPLC with a new 78XP standard. 78XP is too unstable to use as a standard after storage. The results are expressed as the mean ± SD of triplicates.

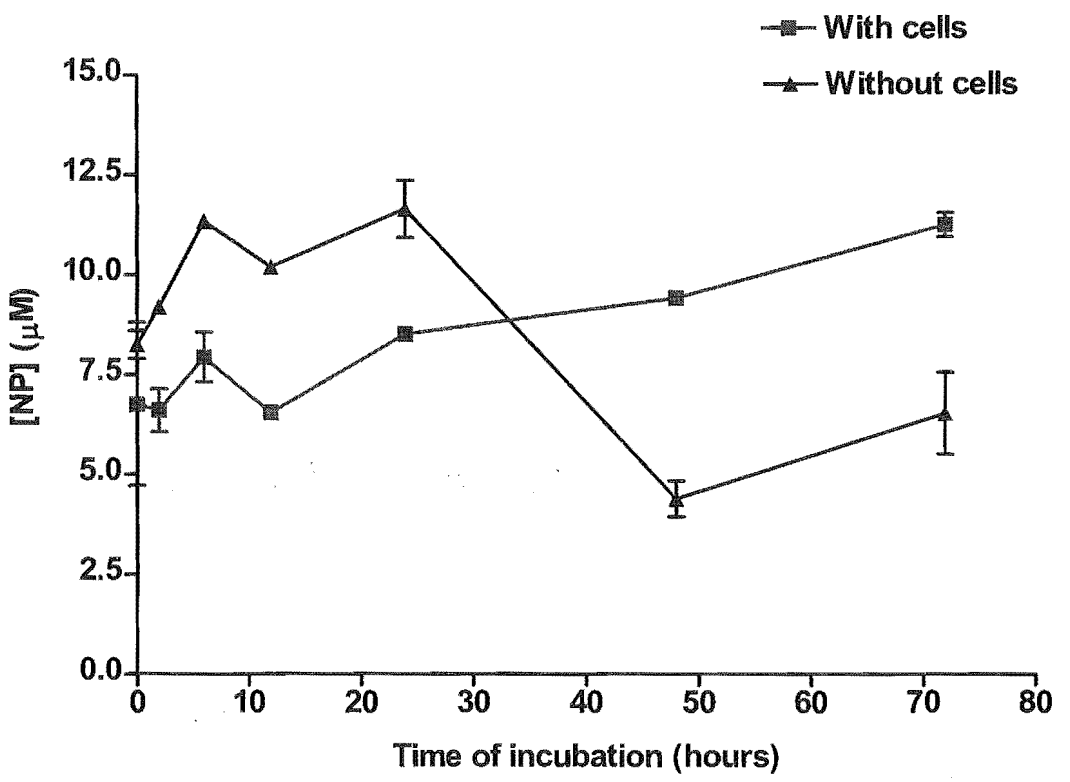

Figure 37: Changes in neopterin concentration during incubation of a 200μM 78NP solution with and without cells over 72 hours. 200μM 78NP was incubated in RPMI 1640 with or without THP-1 cells (5 x 10^5cells/mL) at 37°C for 72 hours. The concentration of neopterin was measured at each timepoint using the pterin HPLC method without oxidation with acidic iodide solution, using the fluorescence detector. The results are expressed as the mean ± SD of triplicates.

2. THE INTERACTION OF 78NP WITH CELLS

In order to examine whether 78NP interacts with cells, 50μM 78NP was added to cells, as in previous viability experiments. After washing, cells were lysed, and levels of remaining 78NP measured.

The control cells showed low levels of 78NP, indicating that they were producing a small amount of the pterin (Figure 38). Both cell types appeared to associate with 78NP, but

the patterns in which this occurred over time were different. Just under 400nM of 78NP was associated with THP-1 cells almost immediately after being added to the wells, and this quantity did not increase over the next ten minutes, which is the time at which oxidants are added in viability experiments. After two hours the concentration of 78NP interacted with was greater. In U937 cells, only approximately 250nM 78NP was associated with the cells immediately after addition to the wells, but this amount had more than doubled by 10 minutes, the time of oxidant addition. The levels of 78NP interacting with the cells fell again over two hours.

78NP is therefore able to interact with cells, in a strong enough way to remain associated with the cells during two sets of centrifugation and PBS washes. The difference between the cell types provides yet another avenue of possibilities to explain the ability of 78NP to protect U937 cells but not THP-1 cells from AAPH and oxLDL. Perhaps the added 78NP interaction at the time of the onset of oxidative stress gives the U937 cells an important advantage in those earlier moments.

This experiment does not differentiate between the possibilities of 78NP being taken into the cell or simply contacting the membrane. A fractionation experiment could give some interesting insights into the nature of the interaction.

To see whether this association alone could be responsible for the protection of the cells from oxLDL, U937 cells were incubated with 200μM 78NP for 20 minutes (Figure 39) or two hours (Figure 40), then washed to remove the 78NP not interacting with the cells before addition of 1.5mg/mL oxLDL, as in other viability experiments.

This type of situation reduced cell viability loss in a study in which U937 cells were preincubated with adult T cell leukemia-derived factor (ADF). ADF is a radical scavenger that is a human homologue of thioredoxin. It still protects cells from TNFα-apoptosis even after it is washed off, reducing the cells' sensitivity to TNFα, perhaps by scavenging radicals, or interacting with molecules on the surface, especially thiol-sensitive proteins such as thioredoxin reductase (Matsuda M. *et al.*, 1991).

In this study, in spite of a small amount of 78NP being present, as illustrated in the previous experiment, no measurable protection of cell viability was provided (Figures 39 and 40). So while the U937 cells' capacity to bind more 78NP early on may give the cells a

headstart against oxidative stress over the THP-1 cells, this could not be the only factor that determines the difference between the two cell lines.

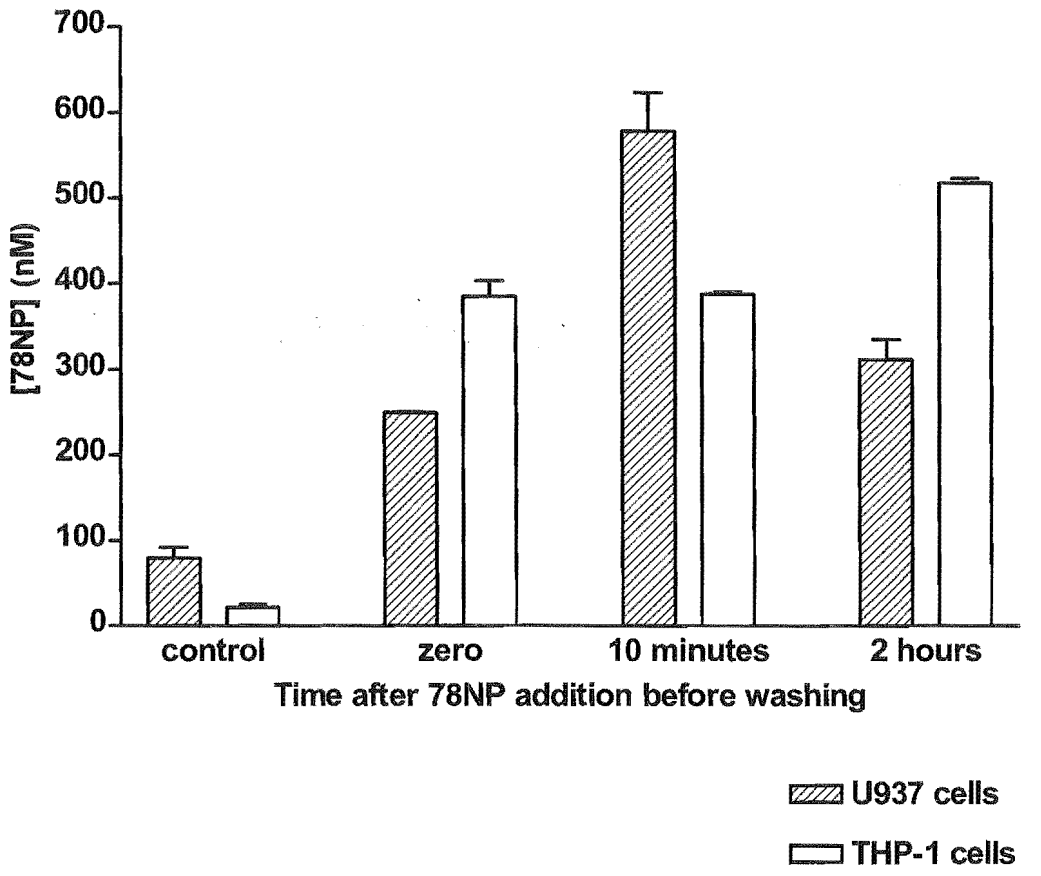

Figure 38: Association between 78NP and THP-1 or U937 cells.
THP-1 cells or U937 cells in RPMI 1640 (5 x 10^5cells/mL) were incubated with 50μM 78NP for up to 2 hours, before the cells were washed twice in PBS and the remaining 78NP measured using the pterin HPLC method. The results are expressed as the mean ± SD of triplicates.

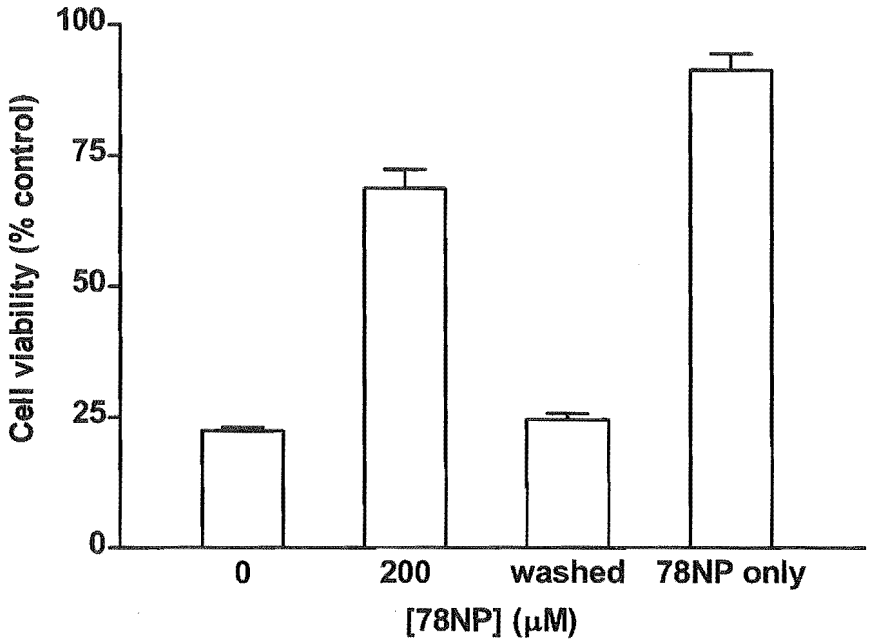

Figure 39: Incubation of U937 cells with 200μM 78NP for 20 minutes and washing before oxLDL addition. U937 cells (5 x 10^5cells/mL) were incubated with 200μM 78NP for 20 minutes then washed twice in PBS and resuspended in RPMI 1640 before 1.5mg/mL oxLDL was added. Controls included cells with oxLDL only, cells with 200μM 78NP only and cells which had 78NP added 10 minutes before the oxidant, which was allowed to remain for the duration of the incubation with the oxLDL. The results were analysed by MTT assay and are expressed as the mean ± SD of triplicates.

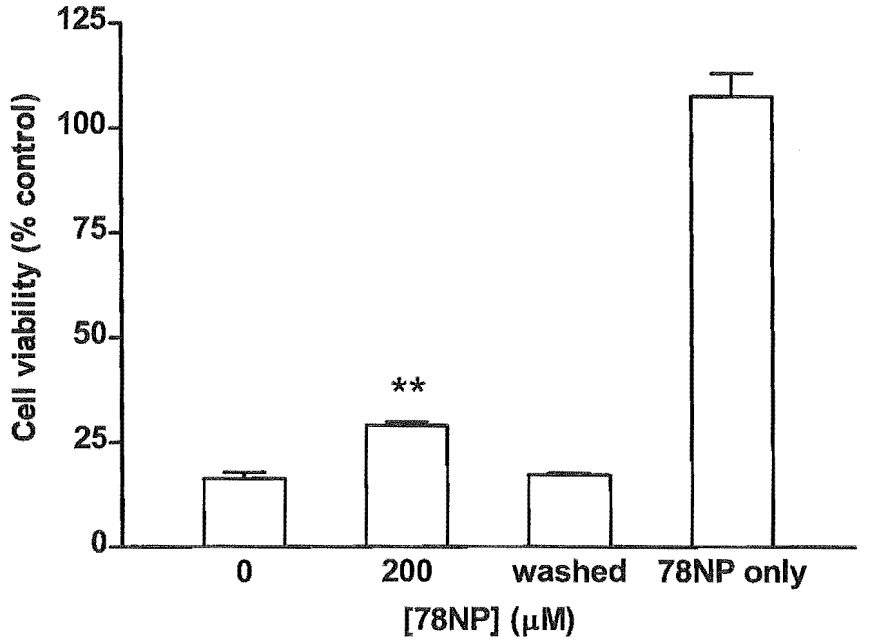

Figure 40: Incubation of U937 cells with 200μM 78NP for 2 hours and washing before oxLDL addition. U937 cells (5 x 10^5cells/mL) were incubated with 200μM 78NP for 2 hours then washed twice in PBS and resuspended in RPMI 1640 before 1.5mg/mL oxLDL was added. Controls included cells with oxLDL only, cells with 200μM 78NP only and cells which had 78NP added 10 minutes before the oxidant, which was allowed to remain for the duration of the incubation with the oxLDL. The results were analysed by MTT assay and are expressed as the mean ± SD of triplicates.

3. THE EFFECT OF 78NP ON OXLDL

It is possible that 78NP's cell viability protecting effect with U937 cells was partially due to an ability to 'detoxify' the oxLDL, perhaps even before it reaches the cells. The oxidative components of the oxLDL may be able to be reduced by the 78NP. Ascorbate and dehydroascorbic acid have been reported to reduce apoptosis mediated by oxLDL in human monocyte-derived macrophages. Ascorbate lowered TBARS and lipid peroxide levels on oxLDL, while dehydroascorbic acid further oxidised or formed adducts with oxidised thiol groups (Asmis and Wintergerst, 1998).

To test whether 78NP can reduce oxLDL's toxicity, 1.5mg of oxLDL in RPMI 1640 was preincubated for 24 hours at 37°C with 78NP at 370μM and 926μM. These concentrations were used so that when the oxLDL and 78NP solution was later added to a well containing THP-1 or U937 cells, the 78NP concentration would be diluted to the 20μM and 50μM concentrations used in earlier cell viability experiments.

As before, 20μM or 50μM of 78NP, added to the cells just 10 minutes before the oxLDL, was unable to reduce the loss of cell viability in THP-1 cells due to the oxLDL (Figure 41). The large amount of protection given by the samples that had the 78NP and oxLDL preincubated together was therefore a result of a process that took place during the preincubation period, when the oxLDL was exposed to a much higher concentration of 78NP.

This possibility is supported by the outcome with the U937 cells, in which 20μM and 50μM of 78NP were able to slightly stop the viability loss mediated by oxLDL, but the preincubated samples had much more success, due to the contact of the oxLDL with the much greater amounts of 78NP before addition to the cells (Figure 42).

Controls were also performed. OxLDL preincubated at 37°C by itself did not degrade by 24 hours, although it did by 42 hours, giving less than half the viability loss. 78NP preincubated alone, and then added to cells 10 minutes before oxLDL, as in other experiments, was still able to reduce U937 cell viability loss, even after 42 hours preincubation. Lower 78NP concentrations preincubated with oxLDL, which once added to the well correspond to 78NP concentrations too low to protect U937 cells, were unable to degrade the oxLDL.

In conclusion, 78NP is able to directly reduce the toxicity of oxLDL, but the concentrations required to do this are much greater than those used in the cell viability experiments. This mechanism is therefore unlikely to be a major contributor to the observed effect of 78NP. If the 78NP was degrading oxLDL in the experiments already presented, THP-1 cells should also have been protected from oxLDL-mediated damage.

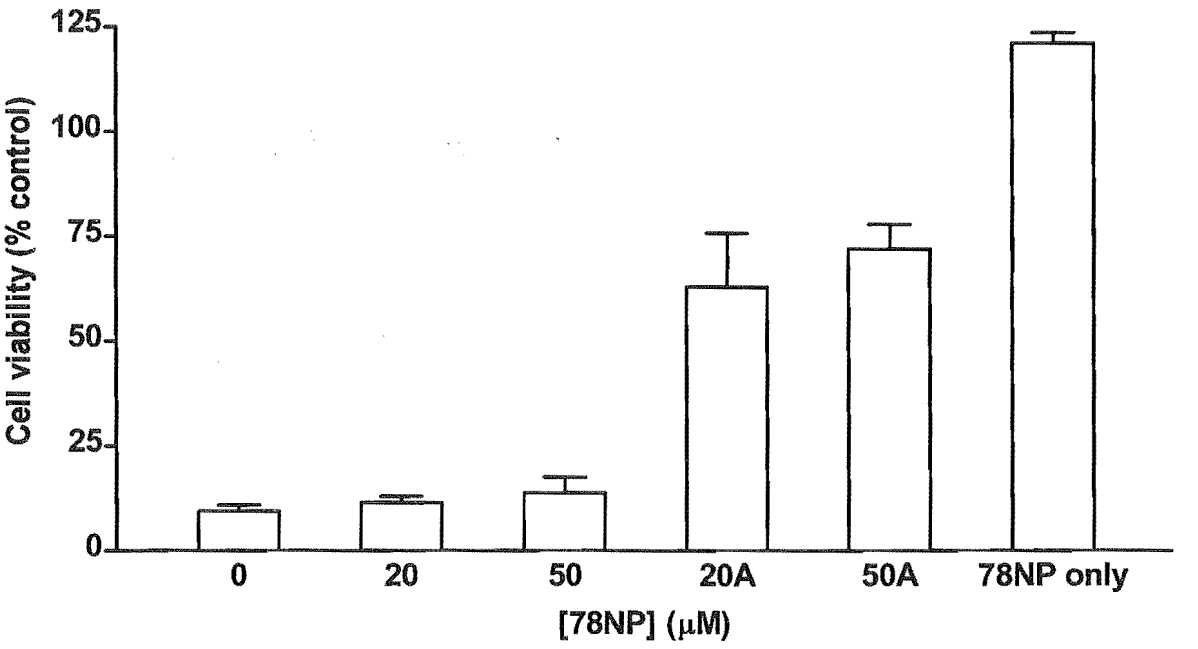

Figure 41: The effect of preincubation of oxLDL with 78NP in THP-1 cells.
The ability of 78NP to degrade oxLDL was tested by incubating 1.5mg of oxLDL with 78NP at 37°C for 24 hours before adding it to the THP-1 cells (5 x 10^5cells/mL). Concentrations of 78NP were selected so that, once added to the cells, they would be diluted to concentrations already tested for effect on cell viability. Here an original concentration of 370μM became 20μM (20A) and 926μM became 50μM (50A). The oxLDL was at 1.5mg/mL. A 200μM 78NP only control was included, and the analysis performed by MTT assay. The results are expressed as the mean ± SD of triplicates.

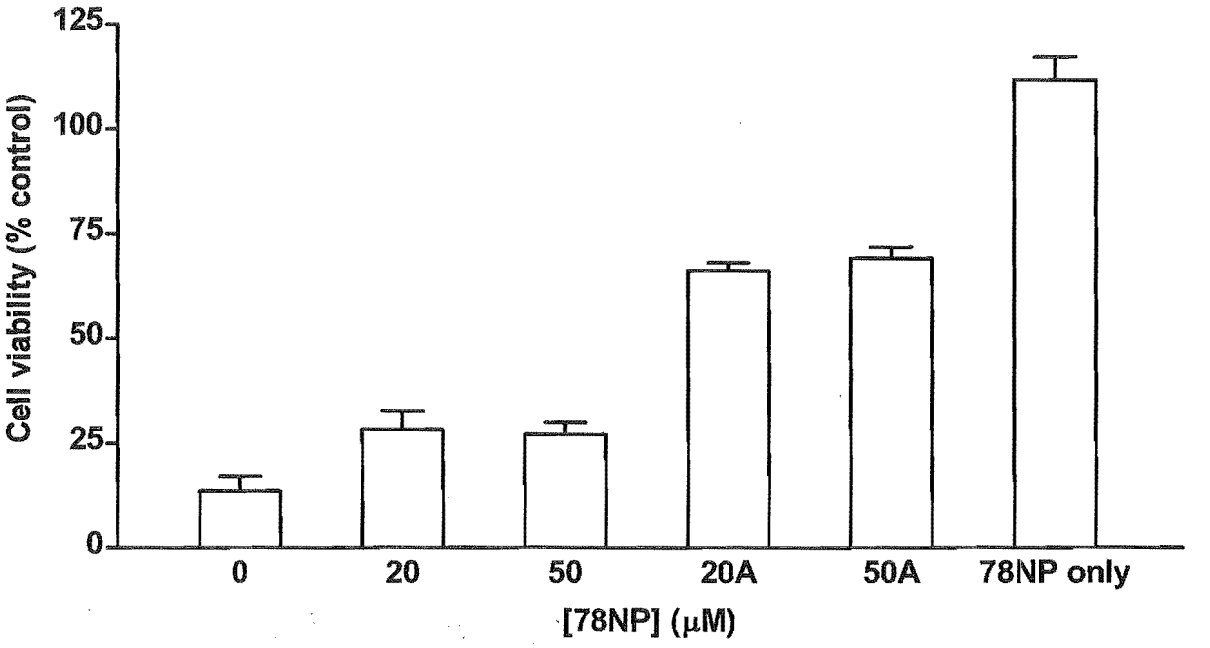

Figure 42: The effect of preincubation of oxLDL with 78NP in U937 cells.
The ability of 78NP to degrade oxLDL was tested by incubating oxLDL with 78NP at 37°C for 24 hours before adding it to the U937 cells (5 x 10^5cells/mL). The experiment was carried out as explained in the previous figure legend. The results are expressed as the mean ± SD of triplicates.

4. THE EFFECT ON THP-1 AND U937 CELLS OF OTHER OXIDANTS AND ANTIOXIDANTS

It is possible that the difference between the two cell types in their reaction to 78NP is not due specifically to 78NP, or even to oxLDL or AAPH, but rather a general reaction of each cell type to oxidants and antioxidants. U937 cells may be able to be protected from oxidants by any antioxidant, whereas THP-1 cells may simply not respond to antioxidants.

These scenarios were both investigated using other oxidants and antioxidants. Two concentrations of ascorbate (25μM and 200μM) were used in Figures 43 and 44 in an attempt to protect the cells against 1.5mg/mL oxLDL. A greater concentration could not be used as it alone caused a loss of cell viability.

Ascorbate was ineffective in both cell types, indicating that the U937 cells are not responsive to simply any antioxidant. Having still not found an efficient antioxidant for THP-1 cells, we cannot be sure that it is possible to protect THP-1 cells from damage by oxLDL

with antioxidants. Ascorbate alone at 200μM did not cause a significant change in cell viability.

Ascorbate has been effective in other cell types with oxLDL. It partially inhibited oxLDL-induced apoptosis in human monocyte-derived macrophages (Asmis and Wintergerst, 1998), and could prevent apoptotic changes caused by 1.5mg/mL oxLDL in human vascular smooth muscle cells, as well as prevent increases in MTT readings and plasma membrane permeability (Siow *et al.*, 1999). Ascorbate did not delay apoptosis in U937 cells incubated with 7-ketocholesterol (Lizard *et al.*, 2000).

An extensive literature search has turned up no examples of THP-1 cells being protected from oxLDL-mediated cytotoxicity by antioxidants. In fact, very few studies have been done looking at antioxidant effect on cytotoxicity with THP-1 cells. Only two found a positive outcome of antioxidants with these cells: N-acetylcysteine could reverse apoptotic effects of diesel exhaust particles in THP-1 macrophages (Hiura *et al.*, 1999), and lipophilic antioxidants such as probucol, vitamin E and BHT could inhibit cell damage by low levels of Cu^{2+} in THP-1 monocytes. A lipophilic chelating agent was required to allow transport of the copper into the cells (Crutchley and Que, 1995).

Ethanol (EtOH) was used as another cellular toxin to test 78NP's capabilities. It is a similar cell death mediator to oxLDL, in that it has a range of effects on cells, some of which involve oxidative stress. EtOH may cause oxidative stress by affecting antioxidant protection systems. It decreased SOD activity in rat brain homogenates and cultures of rat, mouse, hamster and chick neuronal cells. It was shown to increase lipid peroxidation in the livers of rats and baboons, and decrease GSH levels in the liver, brain and kidney cells of those animals (Halliwell and Gutteridge, 1999).

In cell culture, the formation of radicals has been found to be important to ethanol's effects. EtOH enhanced levels of iNOS mRNA in rat alveolar macrophages through the formation of the hydroxyethyl radical (Greenberg *et al.*, 1999). The production of superoxide by PMA-stimulated and unstimulated blood monocyte-derived macrophages, Kupffer cells, sinusoidal endothelial cells and isolated colonocytes was significantly increased by incubation with EtOH (Wickramasinghe, 1989; Grisham *et al.*, 1991; Hasegawa *et al.*, 2002). EtOH stimulation produced hydrogen peroxide in SVEC4-10 cells (Qian *et al.*, 2003) and

catalase and vitamin E prevented EtOH-induced Ca^{2+} rises in vascular smooth muscle cells (Li *et al.*, 2003).

Ethanol has been found to cause increased apoptosis in human and rat monocytes isolated after an alcohol drinking binge (Singhal *et al.*, 1999), in HL-60 cells after incubation with low concentrations of ethanol for four days (Arror and Baker, 1997) and in THP-1 cells, with the concentrations of ethanol and lengths of incubation used here (Brown *et al.*, 1996).

Two concentrations of ethanol, 3% and 6%, incubated with THP-1 cells for 3 hours, caused little loss in cell viability, and a 6 hour incubation period was used for the remainder of the experiments (Figure 45). The reduction in cell viability was similar for both 3% and 6%. Three % ethanol was chosen to test the effect of increasing concentrations of 78NP in THP-1 cells, since 78NP may have a better chance at protecting against a lower ethanol concentration. However, the 78NP was ineffective at all concentrations against the ethanol (Figure 46).

In U937 cells, there was a much larger difference between 3% and 6% at 6 hours (Figure 45). The response to 78NP was tested at both concentrations, and had no effect at either (Figure 47 and 48).

So here is yet another case in which THP-1 cells do not respond to 78NP, and a first instance of U937 cells not being affected by it, demonstrating that the type of oxidant used does actually affect 78NP's protective capacity in U937 cells.

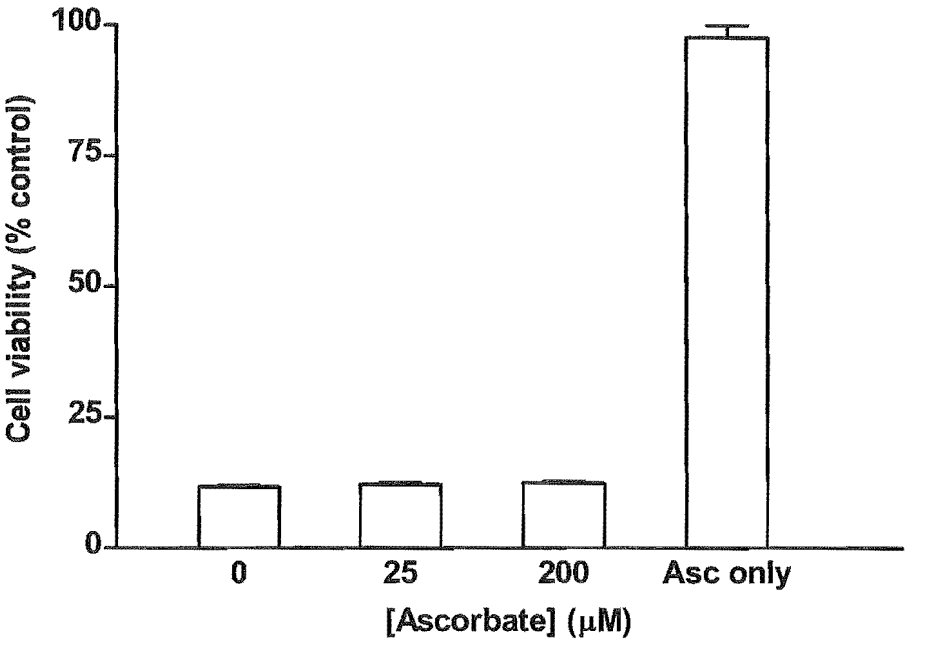

Figure 43: The effect of ascorbate on oxLDL-mediated cell viability loss in THP-1 cells.
THP-1 cells (5 x 10^5cells/mL) in RPMI 1640 were incubated with 1.5mg/mL oxLDL and increasing concentrations of ascorbate for 48 hours. A 200μM ascorbate only control was included. Analysis was performed with the MTT assay. The results are expressed as the mean ± SD of triplicates.

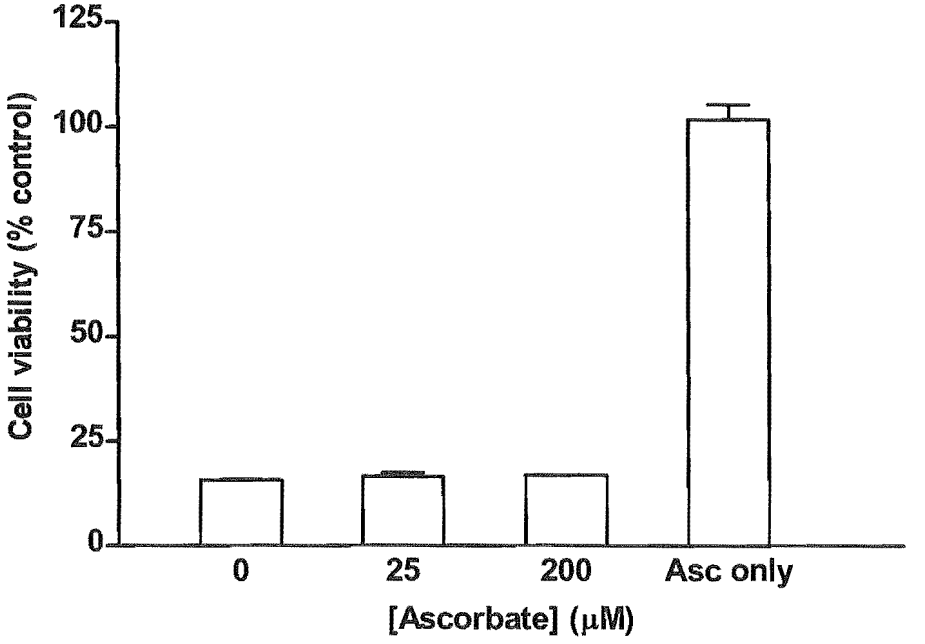

Figure 44: The effect of ascorbate on oxLDL-mediated cell viability loss in U937 cells.
U937 cells (5 x 10^5cells/mL) in RPMI 1640 were incubated with 1.5mg/mL oxLDL and increasing concentrations of ascorbate for 48 hours. A 200μM ascorbate only control was included. Analysis was performed with the MTT assay. The results are expressed as the mean ± SD of triplicates.

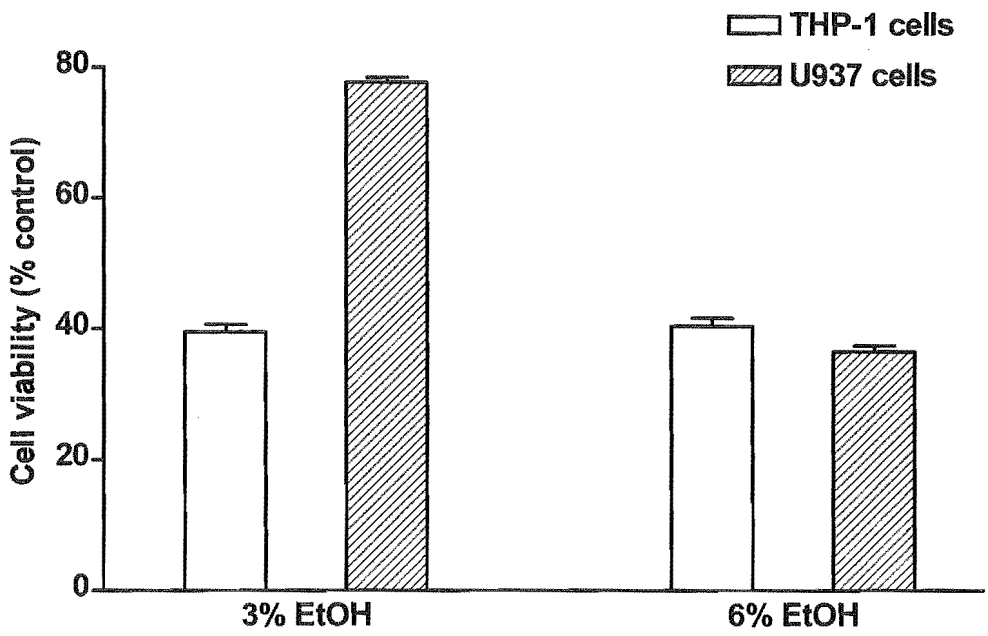

Figure 45: Loss of viability in THP-1 cells and U937 cells in 6 hours with ethanol.
THP-1 or U937 cells (5 x 10^5cells/mL) in RPMI 1640 were incubated for 6 hours with two concentrations of ethanol. The results were analysed by MTT assay, and expressed as the mean ± SD of triplicates.

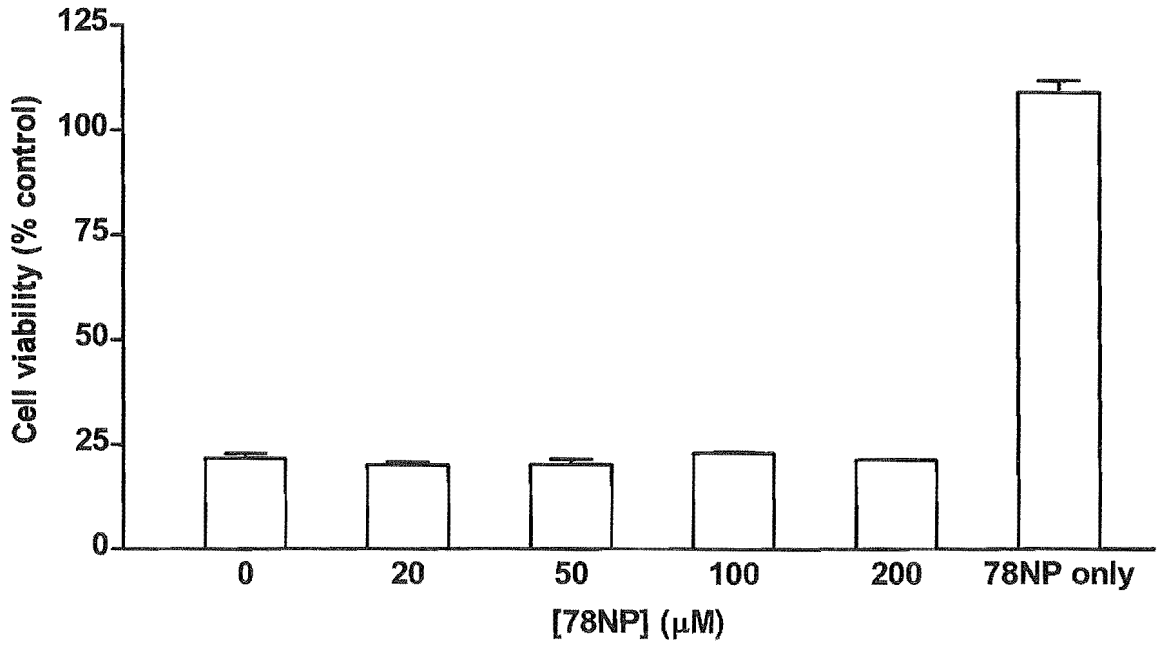

Figure 46: Effect of 78NP on ethanol-induced loss of cell viability in THP-1 cells in 6 hours.
THP-1 cells (5 x 10^5cells/mL) in RPMI 1640 were incubated for 6 hours with 3% ethanol and increasing concentrations of 78NP. A 78NP-only control was included. The results were analysed by MTT assay, and expressed as the mean ± SD of triplicates.

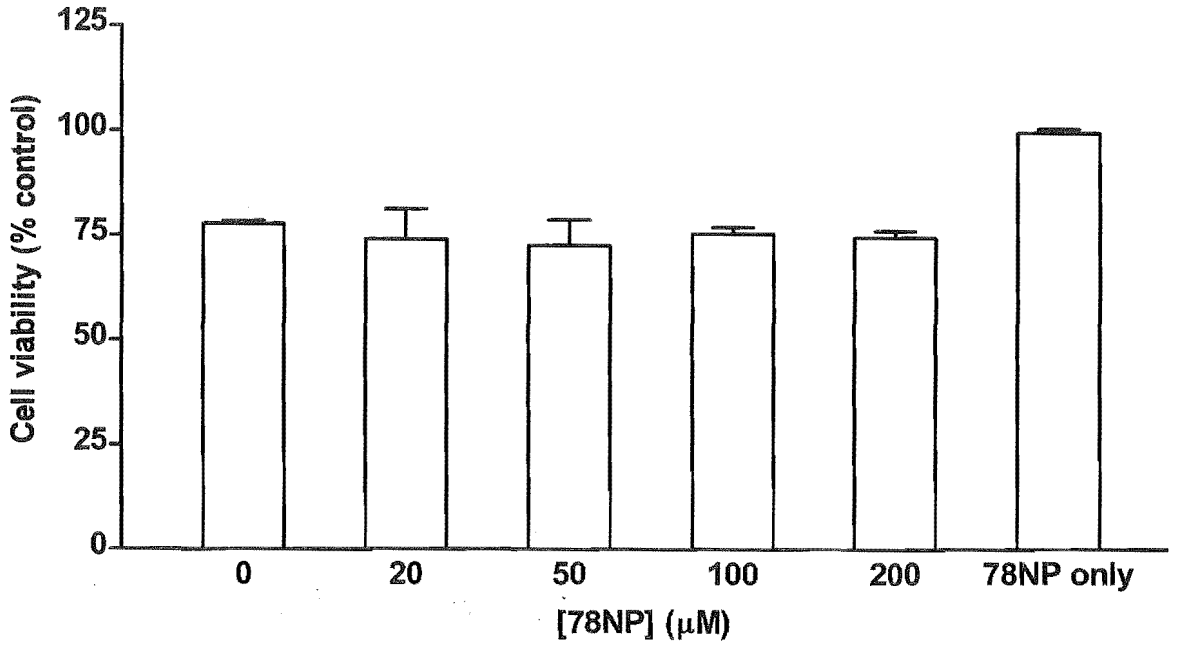

Figure 47: Effect of 78NP on loss of cell viability induced by 3% ethanol in U937 cells in 6 hours. U937 cells (5 x 10^5cells/mL) in RPMI 1640 were incubated for 6 hours with 3% ethanol and increasing concentrations of 78NP. A 78NP-only control was included. The results were analysed by MTT assay, and expressed as the mean ± SD of triplicates.

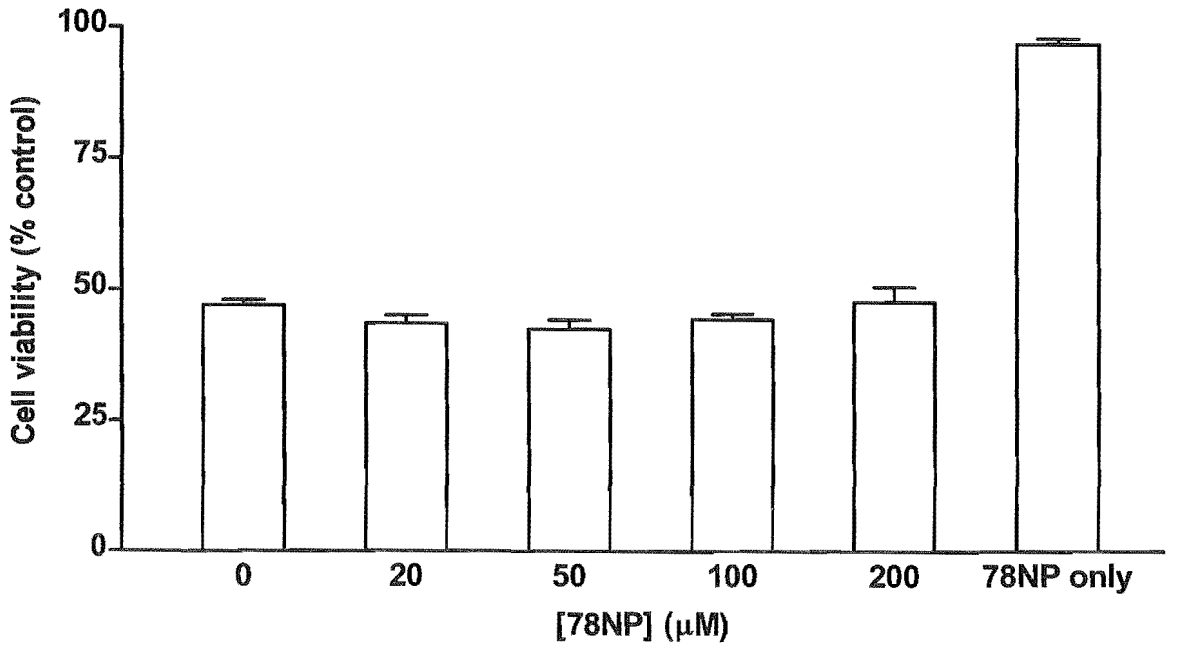

Figure 48: Effect of 78NP on loss of cell viability induced by 6% ethanol in U937 cells in 6 hours. U937 cells (5 x 10^5cells/mL) in RPMI 1640 were incubated for 6 hours with 6% ethanol and increasing concentrations of 78NP. A 78NP-only control was included. The results were analysed by MTT assay, and expressed as the mean ± SD of triplicates.

5. THE EFFECT OF TIME OF 78NP ADDITION ON ITS PROTECTIVE CAPACITY

Previous research from our laboratory has reported that in order for 78NP to have a protective effect on cells, it must be added to the cells between five and ten minutes earlier than the oxidant (Gieseg *et al.*, 1995; Duggan *et al.*, 2002). It was proposed that some sort of equilibrium with the cells must be established for the 78NP to have its effect. Incubation with cells was found to be required in order to obtain reproducible, concentration-dependent results, which was suggested to show an association with the membrane, which may be the point of protection (Duggan *et al.*, 2002).

The necessity of this preincubation was tested by adding 200μM 78NP to wells containing U937 cells in RPMI 1640, two hours or ten minutes before 1.5mg/mL oxLDL, or at the same time as the oxLDL. There was no significant difference between the resulting protective effects for each time of 78NP addition (Figure 49).

The idea of adding 78NP at different times was taken further by adding 78NP to cells incubated with 1.5mg/mL oxLDL in RPMI 1640 at five timepoints during the 48 hour incubation. 78NP was first added to U937 cells ten minutes before the oxLDL was added, as in the usual experimental situation. It was also added 2, 6, 12 and 24 hours after the oxLDL. The 24 hour timepoint was therefore halfway through the total 48 hour incubation (Figure 50).

Late addition of antioxidants has been successful before. In U937 cells incubated with cadmium, antioxidants added after treatment, as well as prior to it, could restore the cells to full viability (Galán *et al.*, 2001).

The protective effect provided by 78NP added two hours after oxLDL was not significantly different to when it was added ten minutes before oxLDL addition (Figure 50). Even when 78NP was added six hours into the incubation with oxLDL, it still appeared to have a small protective capacity, although it was no longer significant. Once twelve hours was reached, the damage already done or set in motion by the oxLDL was unable to be compensated for by the 78NP, and so from this time on, 78NP had no effect. The part or parts of viability loss on which the 78NP has an effect have therefore completely past by twelve hours. They must occur relatively early, as they have taken place for many cells by six hours.

A similar situation has been found in myeloid cells incubated with a range of apoptotic initiators, including cycloheximide, γ-irradiation and p53. Apoptotic cells were not seen until at least eight hours after the start of the incubation, and protection could be provided by protease inhibitors, BHA, IL-6 or IFNγ added at any time up to this eight hour point. The protective effect would still be the same in the case of IFNγ and IL-6, and reduced but still significant in the case of BHA and the protease inhibitors (Lotem and Sachs, 1996).

To help explain this time effect of 78NP, the length of incubation of U937 cells with 1.5mg/mL oxLDL required to obtain a drop in cell viability was examined. A gradual, linear loss of viability over time was found when cells were incubated with oxLDL for between two and 48 hours, and viability was analysed at the end of each timepoint (Figure 51).

If, however, cells were incubated with the oxLDL for between two and 48 hours, and at the end of the designated time, washed twice in PBS to remove the remaining oxLDL, and resuspended in fresh RPMI 1640 for incubation for the remainder of the 48 hour period, the results were different (Figure 52). A small decrease in cell viability was apparent by two hours of incubation with oxLDL. The viability dropped slightly more by six hours. From twelve hours on, the effect was complete. The process the oxLDL had initiated did not require any further input from the oxLDL after this time.

This incubation (or re-incubation) for the whole 48 hours was necessary in order to see the full effects of the oxLDL. Whatever process the oxLDL began, it seemed to require time before its effects were fully visible, at least for the assays used here.

This has implications for the interaction of oxLDL with the cells. If oxLDL is taken up by the cells, the full amount required for maximum effect on viability is not acquired until twelve hours. Alternatively, the oxLDL may need to spend all this time interacting with the membrane from outside the cell, to slowly set the pathways of cell death in motion.

Another example of cell death after removal of the stimulant was found when U937 cells, Jurkat T cells and pre-B leukemia JM1 cells were incubated with cladribine. Apoptosis still occurred after the drug was removed, since commitment to death had already happened. It is probably linked to mitochondrial membrane potential loss (Marzo *et al.*, 2001). With U937 cells incubated with puromycin, inhibitors of cell viability loss were not effective if GSH was already extruded. This represented a committed step in the process of cell death (Ghibelli *et al.*, 1998). This may be the case for U937 cells incubated with oxLDL.

In a similar investigation involving the loss of reduced thiols caused by 1.5mg/mL oxLDL over the 48 hour incubation period (Figure 53), no extra incubation time was required for an effect of oxLDL to be seen. The oxidative effect on thiols was therefore much more immediate. No loss of reduced thiols had occurred at the zero timepoint. By two hours, the reduction was small. By six hours, a little more loss had occurred. From 12 hours onwards no more significant changes took place, and the maximum amount of thiol loss had occurred.

The results shown in the three Figures 50, 52 and 53 fit together well, presenting a picture of the changes occurring in U937 cells over a 48 hour incubation with oxLDL, and how 78NP can affect this. Both cell viability and thiol loss occur gradually at first, reaching their maximum by 12 hours of incubation. 78NP is only able to be effective if added before this 12 hour timepoint. By 12 hours, the outcome of the incubation of oxLDL with the cells has either been achieved, or been irreversibly set in motion. Certainly the loss of reduced thiol components has taken place, and this may perhaps be a first step in the loss of cell viability which is able to be detected by the MTT and trypan blue assays later.

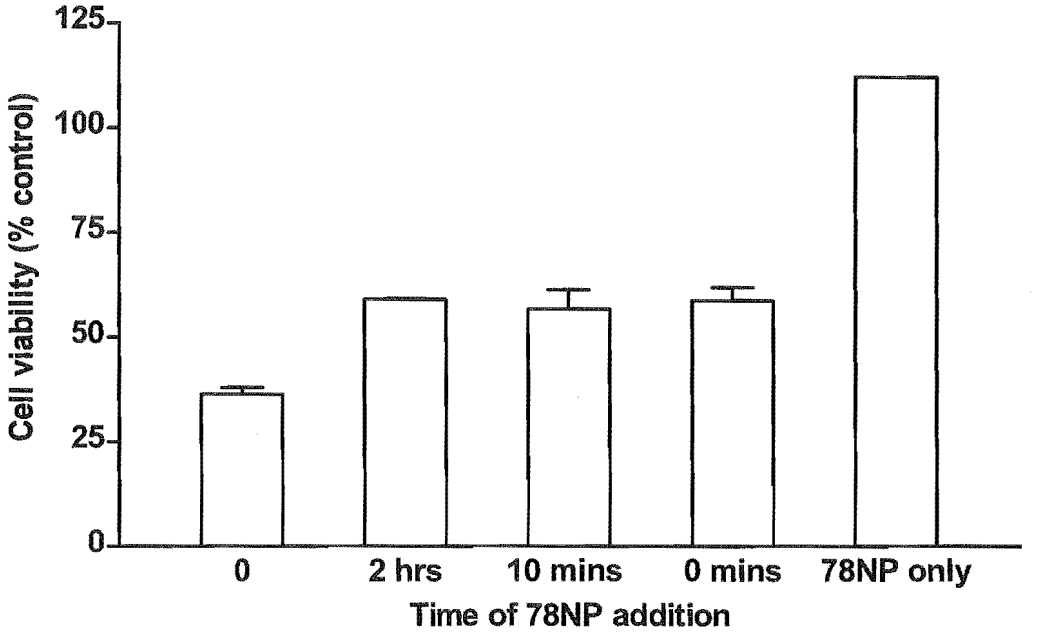

Figure 49: Effect of addition of 78NP at times before oxLDL addition. U937 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 1.5mg/mL oxLDL and 200μM 78NP for 48 hours. The 78NP was added to the cells at three different times: 2 hours or 10 minutes before the oxLDL, or at the same time as the oxLDL. A 200μM 78NP only control was included. The results were analysed by MTT assay, and expressed as the mean ± SD of triplicates.

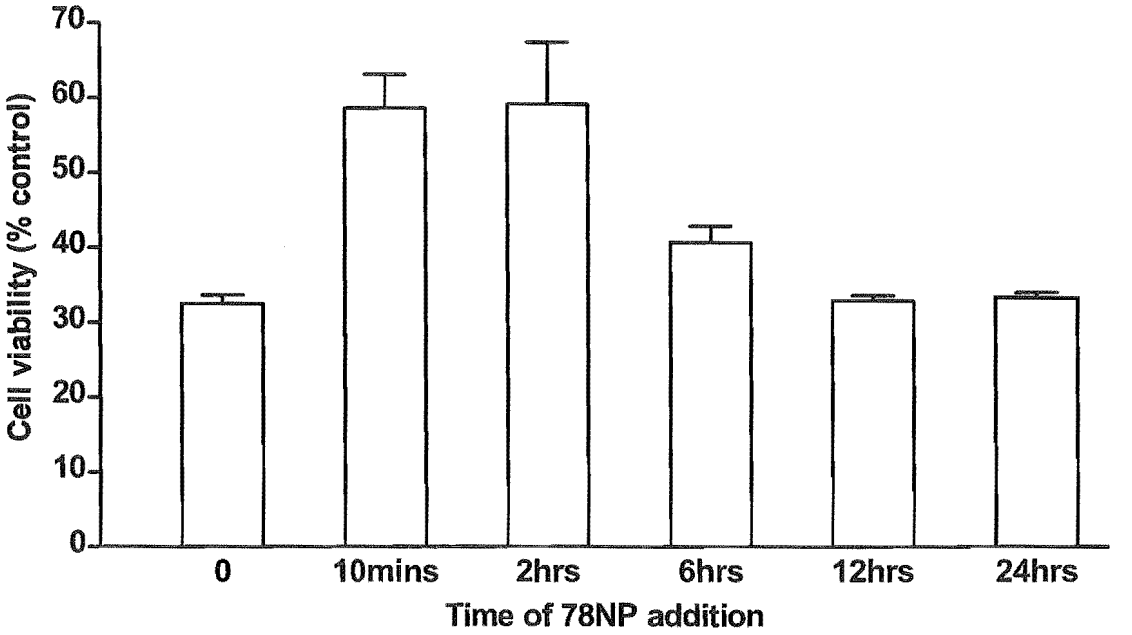

Figure 50: Effect of addition of 78NP at times after oxLDL addition. U937 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 1.5mg/mL oxLDL and 200μM 78NP for 48 hours. The 78NP was added to the cells at five different times: 10 minutes before the oxLDL, or 2, 6, 12 or 24 hours after the oxLDL. The results were analysed by MTT assay, and expressed as the mean ± SD of triplicates.

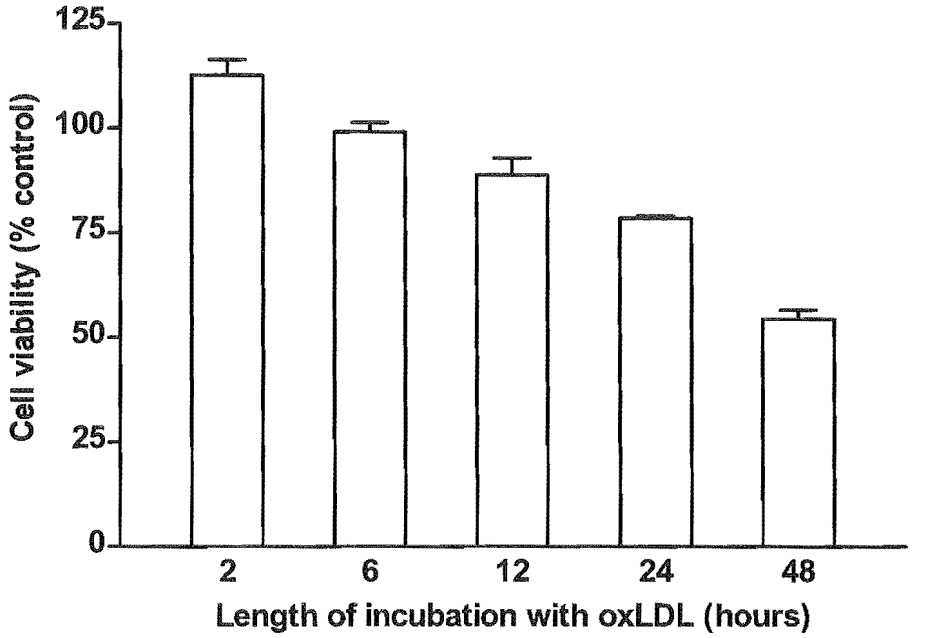

Figure 51: Effect of the length of incubation with oxLDL on cell viability.
U937 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 1.5mg/mL oxLDL for up to 48 hours. After 2, 6, 12 or 24 or 48 hours, the cells were removed from the wells and washed twice with PBS. The results were then analysed by MTT assay, and expressed as the mean ± SD of triplicates.

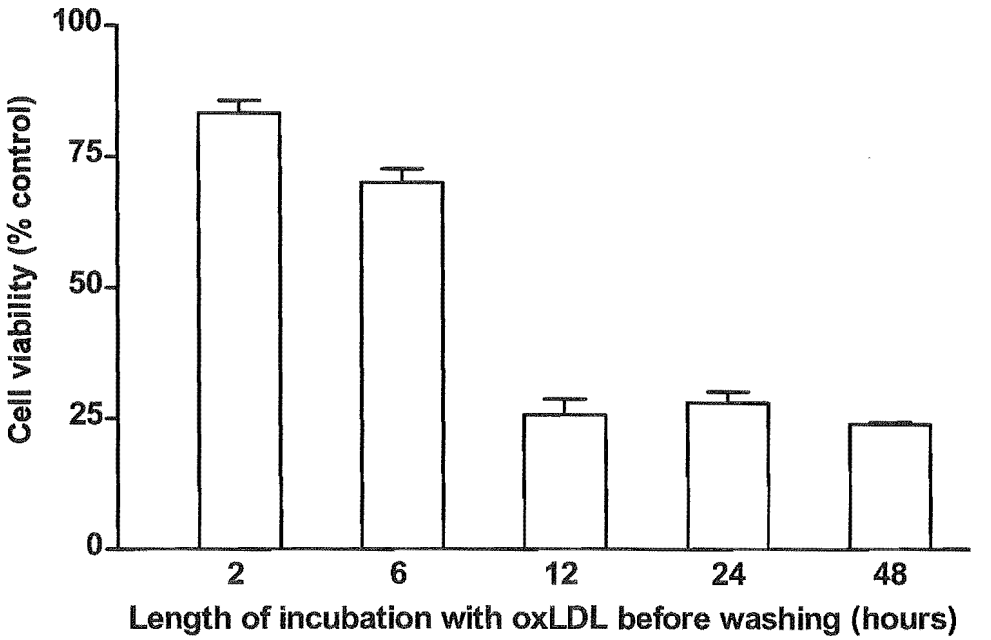

Figure 52: Effect of length of incubation with oxLDL on cell viability after 48 hours.
U937 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 1.5mg/mL oxLDL for up to 48 hours. After 2, 6, 12 or 24 hours, the cells were removed from the wells and washed twice with PBS. They were resuspended in RPMI 1640 and incubated for the remainder of the 48 hours. The results were then analysed by MTT assay, and expressed as the mean ± SD of triplicates.

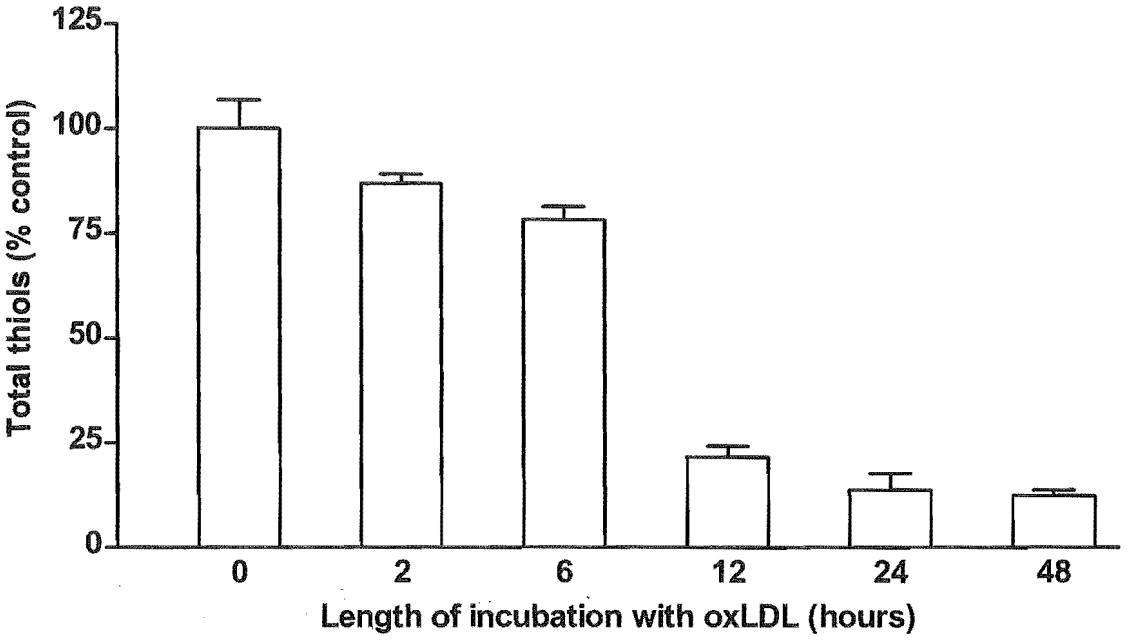

Figure 53: Effect of length of incubation with oxLDL on loss of reduced thiols.
U937 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 1.5mg/mL oxLDL for up to 48 hours. After 2, 6, 12, 24 or 48 hours, the samples were analysed by DTNB assay. The results were expressed as the mean ± SD of triplicates.

C. CELL VIABILITY OF THP-1 MACROPHAGE-LIKE CELLS

The changes that occur during differentiation are said to make PMA-differentiated THP-1 macrophage-like cells a good model for the macrophages that are involved in atherosclerotic plaque formation (Akeson *et al.*, 1991A; Ferret *et al.*, 2000). The effects of AAPH, oxLDL and 78NP on cell viability were tested in this more complex cell system, which may be more relevant to the *in vivo* situation of plaque development.

Cell viability loss by a gradient of AAPH concentrations with THP-1 macrophage-like cells in RPMI 1640 media was concentration-dependent but slight, reaching only 30% viability loss with 50mM of AAPH (Figure 54). The use of EBSS media (Figure 55) resulted in a greater cell viability loss, which appeared to plateau at around 50% of control cell viability, the 10mM and 50mM samples having no significant difference between them.

Increasing 78NP concentrations had no significant effect upon 10mM AAPH-induced viability loss in EBSS (Figure 56). A 200μM 78NP-only control did not cause a change in viability.

Three concentrations of oxLDL were tested with the THP-1 macrophage-like cells for their effect on cell viability in RPMI 1640. Four different incubation lengths were examined. Very little viability loss occurred by 24 hours, and there was no significant difference between the various concentrations, as shown in Figure 57. By 48 hours (Figure 58), 40% viability loss had occurred, but again the loss was not concentration-dependent. At 72 hours, there was an even smaller reduction in viability, as if the cells might be recovering, and again no significant difference between concentrations, as seen in Figure 59. At 96 hours (Figure 60), the 0.5mg/mL oxLDL sample appeared to have fully recovered, having a viability of nearly 100% of the control, while the other two concentrations were still at around 70% of the control, as at the previous timepoint.

Increasing concentrations of 78NP had no apparent effect on the viability loss due to 1.5mg/mL oxLDL at 48 hours (Figure 61), as was seen with the THP-1 monocytes (Figure 25).

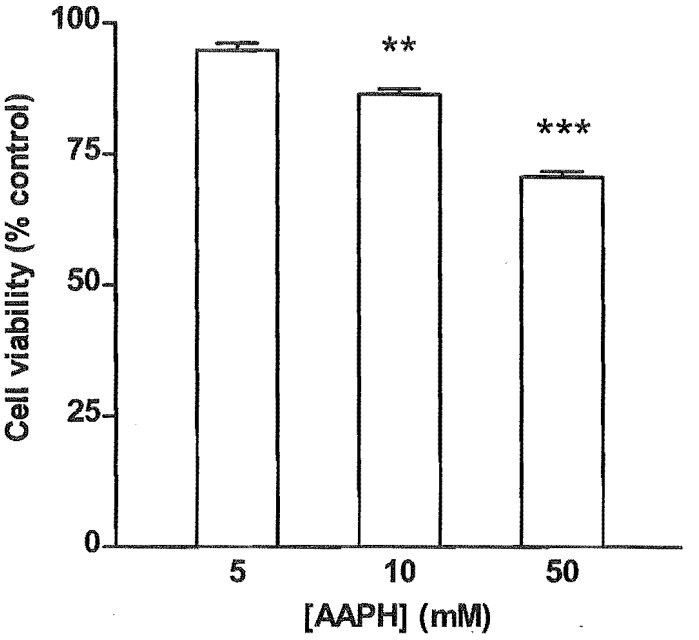

Figure 54: Effect of AAPH on cell viability of THP-1 macrophage-like cells in RPMI 1640.
THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of AAPH in RPMI 1640 for 12 hours. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

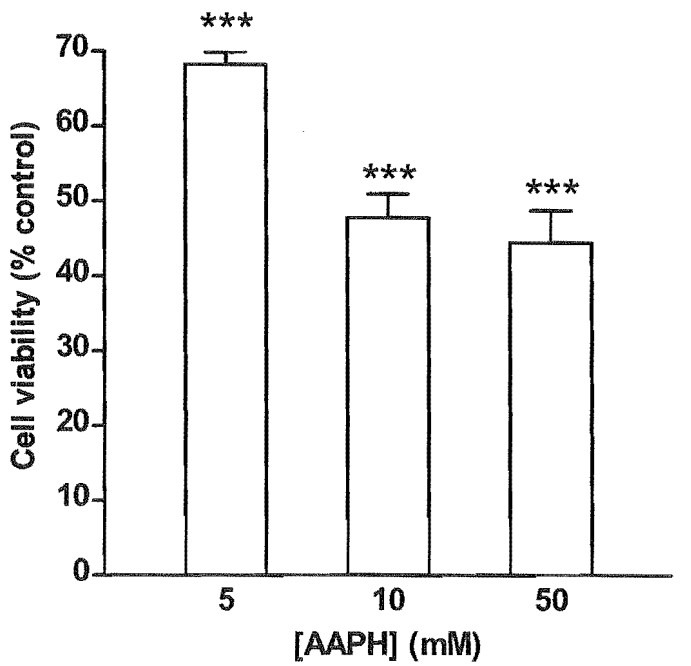

Figure 55: Effect of AAPH on cell viability of THP-1 macrophage-like cells in EBSS.
THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of AAPH in EBSS for 12 hours. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

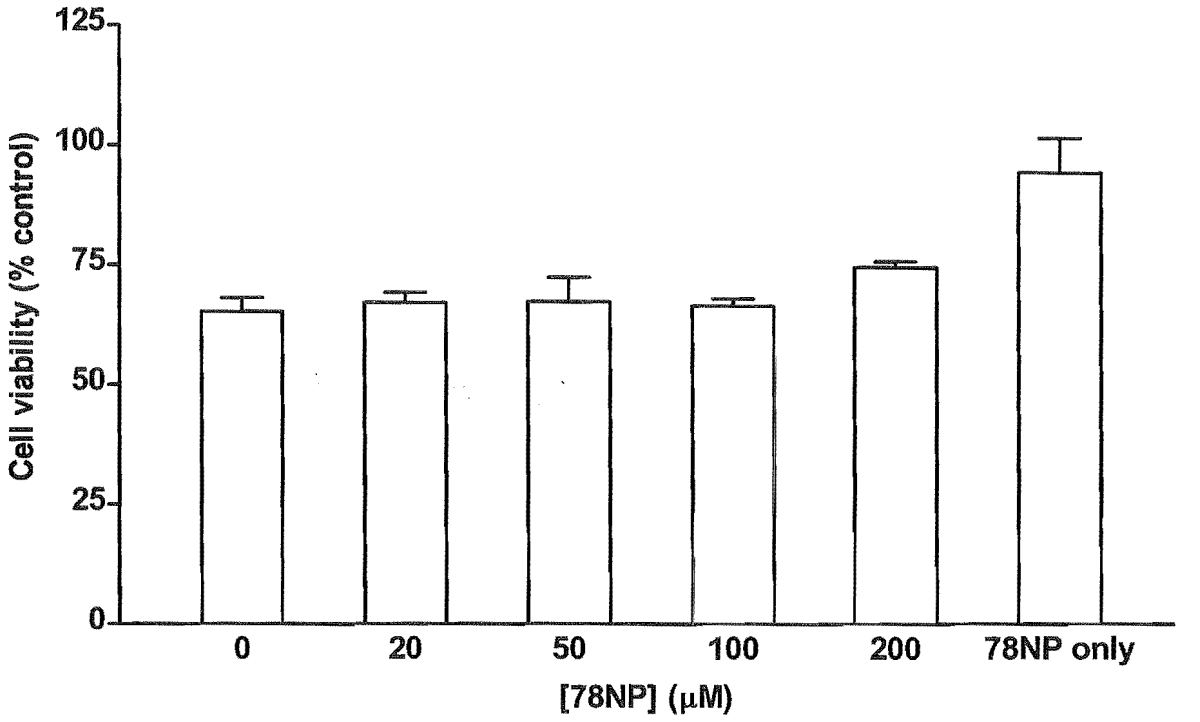

Figure 56: Effect of 78NP on AAPH-mediated loss of cell viability of THP-1 macrophage-like cells in EBSS. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of 78NP and 10mM AAPH in EBSS for 12 hours. A 200μM 78NP-only control was included. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

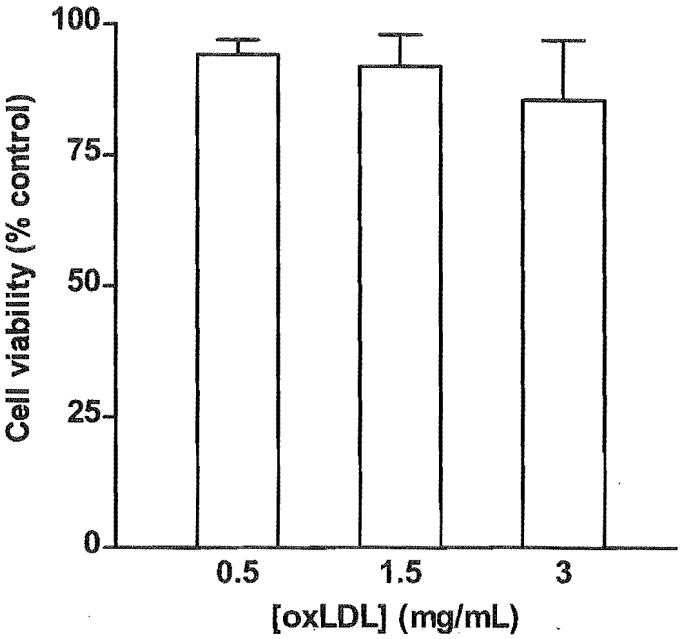

Figure 57: Effect of oxLDL on cell viability of THP-1 macrophage-like cells in RPMI 1640 over 24 hours. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL in RPMI 1640 for 24 hours. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

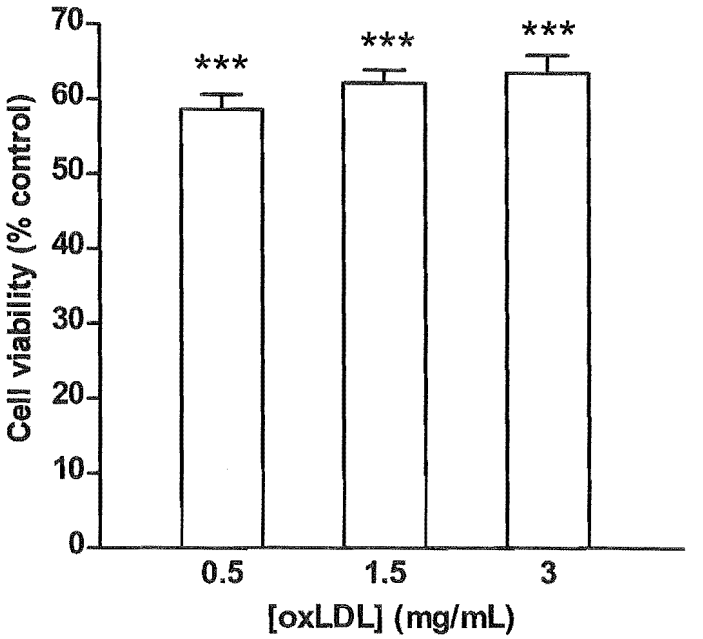

Figure 58: Effect of oxLDL on cell viability of THP-1 macrophage-like cells in RPMI 1640 over 48 hours. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL in RPMI 1640 for 48 hours. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

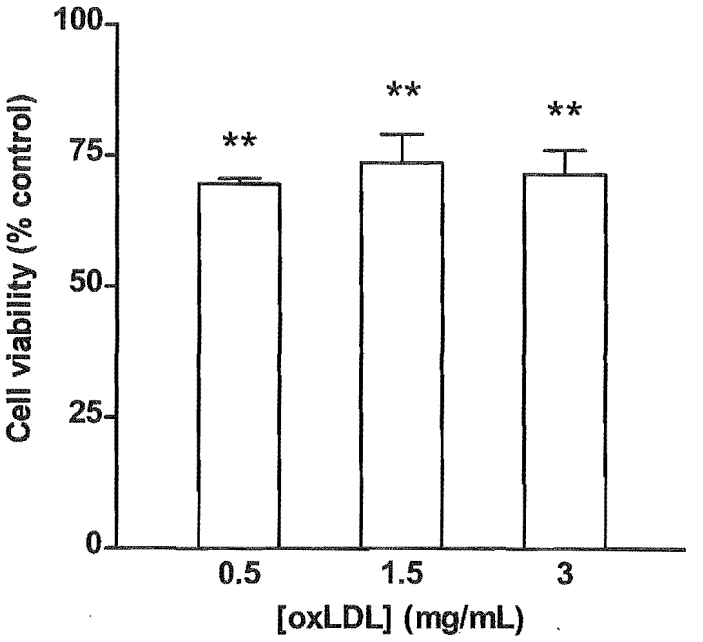

Figure 59: Effect of oxLDL on cell viability of THP-1 macrophage-like cells in RPMI 1640 over 72 hours. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL in RPMI 1640 for 72 hours. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

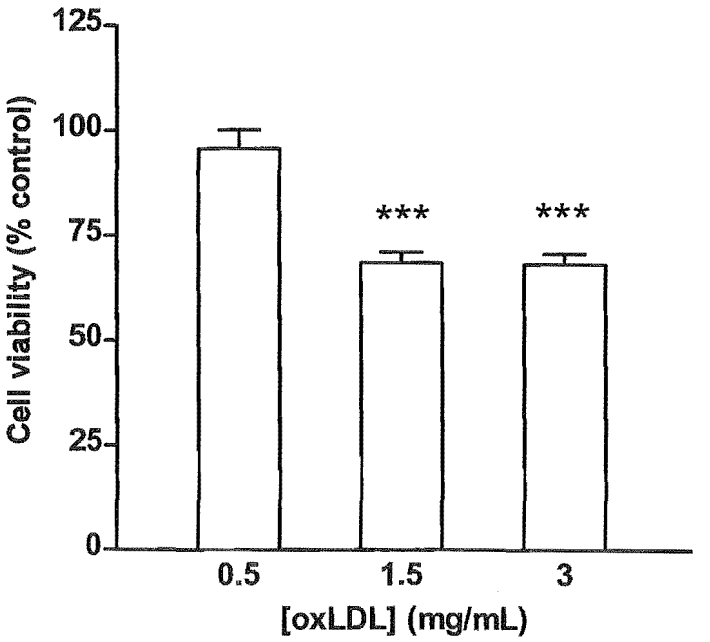

Figure 60: Effect of oxLDL on cell viability of THP-1 macrophage-like cells in RPMI 1640 over 96 hours. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL in RPMI 1640 for 96 hours. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

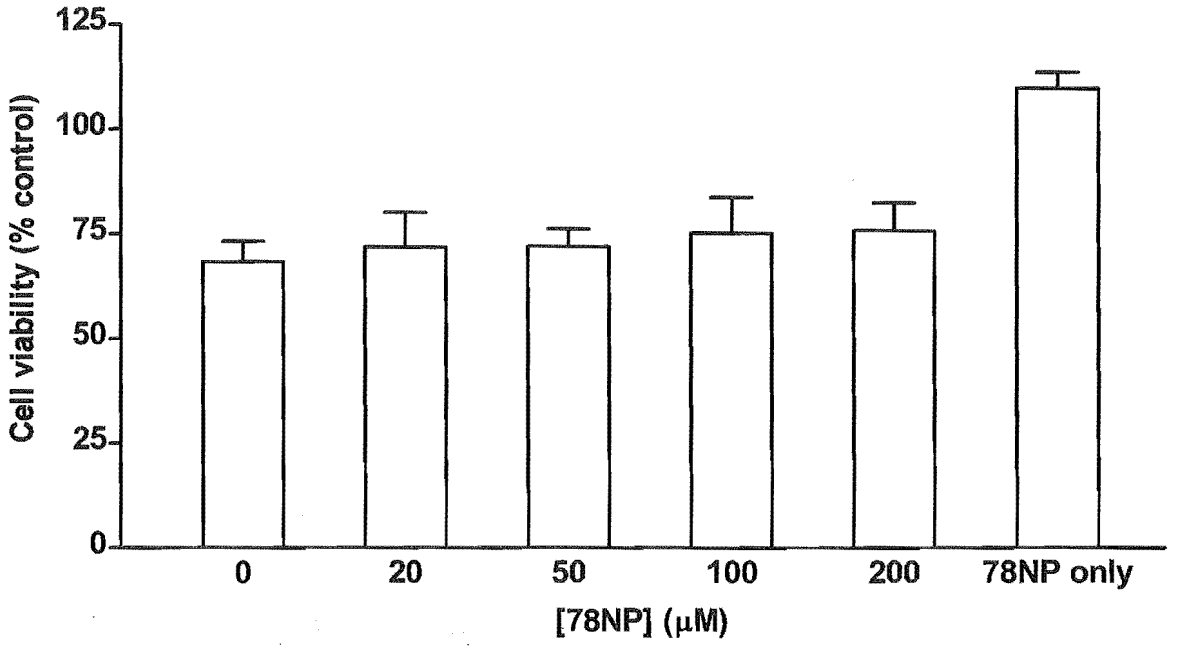

Figure 61: Effect of 78NP on oxLDL-mediated loss of cell viability of THP-1 macrophage-like cells in RPMI 1640. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with increasing concentrations of 78NP and 1.5mg/mL oxLDL in RPMI 1640 for 48 hours. A 200μM 78NP-only control was included. Analysis was performed by MTT assay. Results are expressed as the mean ± SD of triplicates.

Two points of interest arise from these results. Firstly, it appears the differentiated THP-1 cells are hardy and difficult to damage, even with very high AAPH or oxLDL concentrations. They even seem to recover from the insult if left long enough. It should be remembered that there are only a fifth of the number of cells that have been present in previous experiments, even though the concentration of the oxidants remains the same.

The cells' metabolism has been altered by differentiation. For example, lipid metabolism genes become active. Previous research found incubation with acLDL increases cholesterol esterification, although less esterification occurs in THP-1 macrophages than in other cell types. The hydrolysis of the resulting cholesterol esters is also slow (Kritharides *et al.*, 1998). A similar degree of oxLDL accumulation was found in both THP-1 monocytes and PMA-stimulated THP-1 cells, but the PMA enhanced the amount of degradation of the oxLDL (Lougheed *et al.*, 1991).

The secondary products of macrophages are also produced. This includes the secretion of nitric oxide, IL-1β, TNFα, M-CSF, CSF-1, tissue factor VIIa, lipoprotein lipase and apoE, and the transcriptional activation of *c-fos* and *c-jun*, collagenase, phospholipase A_2 and human metallothionein IIA (Auwerx *et al.*, 1989; Via *et al.*, 1989; Akeson *et al.*, 1991B; Liu and Wu, 1992; Schwende *et al.*, 1996; Kritharides *et al.*, 1998; Mouithys-Mickalad *et al.*, 2001). The cells are also able to generate an oxidative burst, activating NADPH oxidase. Superoxide production increases tenfold with stimulation (Schwende, 1996). OxLDL is one possible stimulant (Nguyen-Khoa *et al.*, 1999).

The proportions of antioxidants change with the cells' new oxidative environment. THP-1 monocytes were reported to have a threefold lower glutathione level than THP-1 macrophages that had been incubated with PMA for 48 hours (Gotoh *et al.*, 1993). Another study found THP-1 macrophage-like cells expressed 30% more Cu/ZnSOD, 40% more MnSOD, 70% more thioredoxin and 120% more thioredoxin reductase, but had no increase in catalase or glutathione (Ferret *et al.*, 2000).

Because of these changes, it is not surprising that THP-1 macrophage-like cells have an altered response to AAPH and oxLDL, becoming more robust and long-lived. THP-1 macrophage-like cells have been previously shown to have increased tolerance to ionizing radiation (Munn *et al.*, 1995), and did not experience the same level of hydrogen peroxide induced apoptosis as the THP-1 monocytes (Ferret *et al.*, 2000).

THP-1 cells differentiated with PMA were found to be very resistant to 5.0mg/mL oxLDL in a study examining phosphatidylserine exposure and propidium iodide uptake after 24 hours, with less than 10% of cells undergoing apoptosis (Vicca *et al.*, 2000). The same cell type underwent limited cell death with 1.0mg/mL oxLDL or 20μg/mL 7-ketocholesterol over 24 hours and this was halved in cells with greater expression of the class A scavenger receptor (Liao *et al.*, 2000).

This effect has also been found in other cell types. Cells of the rat pheochromocytoma cell line PC12 were less sensitive to pterin or 5-azacytidine-induced apoptosis after differentiation, and PHA-differentiated peripheral blood mononuclear cells underwent delayed cell death with oxLDL. It is important to note that contrary results have also been reported, with some authors finding neurons more sensitive after differentiation, depending on cell type and agent (Alcouffe *et al.*, 1999; Enzinger *et al.*, 2002).

Other responses to oxLDL have also been reportedly altered due to cell differentiation. PPARγ, which regulates genes during differentiation, and human ferritin light chain expression were found to be decreased in THP-1 cells incubated with PMA, in spite of an increase in transcription (Jang *et al.*, 1999). Other studies, which only examined mRNA levels, have described upregulation of nuclear receptors including LXRα, RXRγ and PPARγ by oxLDL in THP-1 cells stimulated by PMA (Ricote *et al.*, 1998; Mikita *et al.*, 2001). Extensively oxidised LDL reduced TNFα levels in THP-1 macrophages, also contrary to findings in undifferentiated cells. A drop in mRNA was found, but part of the decrease might have been due to oxidative modification of the TNFα so that it did not show up with the ELISA method used (Girona *et al.*, 1997).

A reduced reaction to oxLDL toxicity can perhaps be anticipated, since oxLDL stimulates protein kinase C in monocytes, but PMA treatment downregulates it (Deigner and Claus, 1996; Schwende, 1996). A possible reduced upregulation of PPARγ and a lowering of TNFα in differentiated cells may also alter the expected effects of oxLDL on the cell (Girona *et al.*, 1997; Jang *et al.*, 1999).

The differentiation process involves the development of much better defenses, and an altered response to oxidative stress, including the activation of lipid metabolism. In the case of oxLDL, it was suggested that the increased scavenger receptor expression ensures more degradation of the oxLDL, reducing its toxicity (Lougheed *et al.*, 1991; Liao *et al.*, 2000). The lipid changes and scavenger receptors are also affected by cell density. The density used in this set of experiments is low, and would result in cholesterol accumulation, esterification and scavenger receptor expression that are relatively high, which would lower the cells' viability loss from oxLDL (Rodriguez *et al.*, 1994).

The increase in endogenous antioxidants during differentiation would have a role against oxidative effects of oxLDL and peroxyl radicals from AAPH. The possible increases in glutathione and thioredoxin would be important, since both oxLDL and AAPH were shown to lower reduced thiol levels (Gotoh *et al.*, 1993; Ferret *et al.*, 2000). Thioredoxin, being present on the cell surface (Nishiyama *et al.*, 1999), may be effective against AAPH or any extracellular oxidative effects of oxLDL.

The macrophage-like cells might also generate their own oxidants, through the oxidative burst, for example, as a result of stimulation by oxLDL (Nguyen-Khoa *et al.*, 1999). This might modify the oxLDL particle before uptake, altering its toxicity. The toxicity of the oxLDL might also be reduced by the cells' adherence to the bottom of the well-plate, which could restrict access to the cells for the oxidants.

In the same way, this would restrict access to the cells for exogenous 78NP, which may partly account for the lack of effect of the 78NP in protecting the cells from the oxidative damage that does occur. There are other possible reasons for the 78NP's lack of effect on cell viability. If the oxLDL is being processed in a different way from monocytic cells, for example in a different part of the cell, 78NP may not be in the right place in the cell to combat this damage. Also, the THP-1 macrophage-like cells may still possess the characteristics of the THP-1 monocytes which caused 78NP to be ineffective. The results with this macrophage cell type do not provide any evidence that 78NP is able to protect cells from oxLDL-mediated cell death in the atherosclerotic plaque.

SUMMARY

OxLDL caused concentration-dependent viability loss to a similar degree in both THP-1 and U937 monocytes. While THP-1 cells showed an equivalent loss of reduced thiols, U937 cell thiol loss was marked by a sudden and complete depletion after a critical timepoint.

78NP protected U937 cells from the decrease in viability and reduced thiols, but neither of these effects were apparent with the THP-1 cells. This is likely to reflect a difference in cellular metabolism of oxLDL, and its influence on total reduced thiols.

Several insights were provided into how 78NP may act to protect U937 cells from oxidants, while at the same time not protecting THP-1 cells, and how it may interact with the cells. 78NP oxidised over 72 hours to 78XP. The inclusion of cells in the solution stabilised the 78NP, slowing this process. 78NP was able to interact with both cell types, although to a different degree with each over time. The actual amount of 78NP which became associated with the cells was not enough to protect them itself, so it is difficult to know if this interaction is an important one in terms of the survival of the cells.

78NP was able in some way to degrade or 'detoxify' oxLDL during an incubation of 24 hours, but the concentrations required for this were much larger than those used in experiments, so this is unlikely to be a significant mechanism in the protection of cells from oxLDL.

Tests using both cell types with oxLDL and ascorbate, or ethanol and 78NP showed that U937 cells cannot be protected from cell viability loss by every antioxidant, and that 78NP cannot protect U937 cells against all stresses. THP-1 cells have not yet been shown to be protected against oxLDL by an antioxidant.

The effect of the addition time of the 78NP depends on the timing of cellular responses caused by the oxidant. The time of 78NP addition to an oxLDL assay system did not affect the result for the first two hours of the incubation. It still had some effect if added six hours later, but from twelve hours, no protective capacity was evident. Twelve hours was also the time at which the oxLDL had first caused the maximum amount of thiol loss, and the process resulting in the maximum amount of cell viability loss had been irreversibly begun. Commitment to cell death must have occurred by this point.

PMA-differentiated THP-1 macrophage-like cells were very resistant to cell viability loss with AAPH and oxLDL, even at high concentrations and over long periods of time. This may be due to the changes in cell metabolism that have occurred as a result of the differentiation process. 78NP was not effective against cell viability loss caused by either AAPH or oxLDL. This may also be a result of the changes of differentiation, or the continuing possession of the properties which did not allow THP-1 monocytes to be protected by 78NP from AAPH and oxLDL.

MONOCYTES, OXLDL AND APOPTOSIS

INTRODUCTION

These studies characterise the induction of apoptosis by oxLDL in U937 and THP-1 monocyte cells. The effect of 78NP on the development of apoptotic features is examined.

Apoptosis in the plaque is thought to be caused by oxLDL and other factors of inflammation such as TNFα (Farber *et al.*, 1999). Apoptosis may inhibit intimal thickening, limit cell accumulation, counteract mitosis and cause very little inflammation, and would therefore be anti-atherogenic. However, the death of smooth muscle cells, increased release of matrix-digesting enzymes and accumulation of lipids and Ca^{2+} may promote rupture and calcification (Geng *et al.*, 1997).

OxLDL is reported to cause apoptosis in most studies examining its effects on cell viability. This encompasses a wide range of cell types, including macrophage and monocyte cell types such as $P388D_1$ cells (Reid *et al.*, 1993A), J774 cells (Yuan *et al.*, 2000), J774.A1 monocytes and macrophages (Yang *et al.*, 1996), mouse peritoneal macrophages (Niu *et al.*, 1996), mouse macrophages (Liu *et al.*, 1997A), THP-1 monocytes (Vicca *et al.*, 2000), human blood peripheral mononuclear cells (Kinscherf *et al.*, 1998) and human monocyte-derived macrophages (Asmis and Wintergerst, 1998). Endothelial cells are also represented (Dimmeler *et al.*, 1997; Kinoshita *et al.*, 1999; Rusinol *et al.*, 2000), including human endothelial cells (Escargueil-Blanc *et al.*, 1997), human coronary artery endothelial cells (Li *et al.*, 1998) and HUVECs (Galle *et al.*, 1999). Smooth muscle cell types looked at include human vascular smooth muscle cells (Siow *et al.*, 1999), rabbit aortic smooth muscle cells (Nishio *et al.*, 1996) and human arterial smooth muscle cells (Björkerud and Björkerud, 1996). Other cell types studied include cultured lymphoblastoid cells (Escargueil *et al.*, 1992), Jurkat T cells (Alcouffe *et al.*, 1999), mesangial cells (Sharma *et al.*, 1996), rat dorsal root ganglion cells (Papassotiropoulos *et al.*, 1996), neuronal cultures (Keller *et al.*, 1999), lung fibroblasts (Björkerud and Björkerud, 1996), Chinese hamster ovary cells and thymocytes (Rusinol *et al.*, 2000).

Both heavily and lightly oxLDL are reported to cause apoptosis (Escargueil *et al.*, 1992; Reid *et al.*, 1993A), however most authors do not characterise their oxLDL further than stating that it is oxidised. The majority of studies use Cu^{2+}-oxidised LDL, although one paper compares Cu^{2+}- and HOCl-oxidised LDL, determining that the former had higher TBARS and carbonyls and the latter more protein carbonyls and advanced oxidation protein products. With THP-1 monocytes, the Cu^{2+}-oxidised LDL was reported to induce a stronger apoptotic response (Vicca *et al.*, 2000).

Concentrations of oxLDL purported to cause apoptosis vary, ranging from 0.1mg/mL to 1.0 mg/mL in various human endothelial cells (Escargueil-Blanc *et al.*, 1997; Li *et al.*, 1998; Galle *et al.*, 1999) and 0.2mg/mL with mouse macrophages (Niu *et al.*, 1996; Liu *et al.*, 1997) to 1.25mg/mL and 5.0mg/mL with THP-1 monocytes (Vicca *et al.*, 2000).

Timeframes for the experiments looking at oxLDL effects are relatively long, running from 24 to 48 hours, independent of cell type or oxLDL concentration (Escargueil *et al.*, 1992; Niu *et al.*, 1996; Yuan *et al.*, 2000). Studies that only considered changes up to 24 hours were performed on Jurkat T cells (Alcouffe *et al.*, 1999), rat dorsal root ganglion cells (Papassotiropoulos *et al.*, 1996) and THP-1 monocytes (Vicca *et al.*, 2000).

The studies here examine oxLDL-induced apoptosis in two monocyte cell lines, one of which, U937 cells, has not previously been examined for apoptotic characteristics in the presence of oxLDL. The cells are incubated with concentrations of extensively oxLDL known to cause viability loss over 48 hours, a period of time likely to be sufficient for the apoptotic process to be completed and secondary necrosis to begin, as indicated by trypan blue staining.

OxLDL appears to cause the full spectrum of well-recognised apoptotic markers. Most studies look at only a limited set of features, usually between one and five, mostly two or three. While this may be adequate to identify the cells' condition as apoptotic (depending on which assays are used), it causes difficulties in relating one set of experimental conditions to another.

The most popular way of assessing the presence of apoptosis is oligonucleosomal laddering on gels, showing DNA fragmentation. This was observed with oxLDL in $P388D_1$ cells (Reid *et al.*, 1993A), mouse macrophages (Liu *et al.*, 1997), THP-1 monocytes

(Björkerud and Björkerud, 1996), cultured lymphoblastoid cells (Escargueil *et al.*, 1992), endothelial cells (Dimmeler *et al.*, 1997; Escargueil-Blanc *et al.*, 1997; Lizard *et al.*, 1999), smooth muscle cells (Björkerud and Björkerud, 1996; Nishio *et al.*, 1996; Lizard *et al.*, 1999; Siow *et al.*, 1999), lung fibroblasts (Björkerud and Björkerud, 1996) and Chinese hamster ovary cells (Rusinol *et al.*, 2000).

Other typical nuclear features were also found after incubation with oxLDL. TUNEL, which labels single strand breaks in the DNA strand (Nishio *et al.*, 1996), an earlier phase of DNA fragmentation, gave positive results in J774 cells (Yuan *et al.*, 2000), rat dorsal root ganglion cells (Papassotiropoulos *et al.*, 1996), smooth muscle cells, endothelial cells (Nishio *et al.*, 1996; Lizard *et al.*, 1999) and Chinese hamster ovary cells (Rusinol *et al.*, 2000).

Chromosome margination, or pyknosis, was found in J774 cells (Yuan *et al.*, 2000), human arterial smooth muscle cells, lung fibroblasts and THP-1 monocytes (Björkerud and Björkerud, 1996), and chromosome condensation was shown in human arterial smooth muscle cells, lung fibroblasts and THP-1 monocytes (Björkerud and Björkerud, 1996).

The other most commonly examined apoptotic feature is caspase activation. Caspase enzymes were activated as a result of oxLDL in J774 cells (Yuan *et al.*, 2000) and endothelial cells (Dimmeler *et al.*, 1997). Caspase inhibitors reduced apoptosis and so indirectly proved a role for caspases in the apoptosis of THP-1 monocytes (Vicca *et al.*, 2000), bovine endothelial cells (Farber *et al.*, 1999), endothelial cells, smooth muscle cells (Lizard *et al.*, 1999) and neuronal cultures (Keller *et al.*, 1999) incubated with oxLDL.

Many other apoptotic characteristics have been shown to be caused by oxLDL. However, most were not tested for in more than one or two cases. PARP activation was identified in cultured lymphoblastoid cells (Escargueil *et al.*, 1992), and Fas protein expression was increased by oxLDL in human coronary artery endothelial cells (Li *et al.*, 1998), whereas Fas pathway activation was not required for oxLDL-mediated apoptosis in macrophages (Yao and Tabas, 2001). Ceramide increased in human monocytes, bovine endothelial cells and human endothelial cells (Kinscherf *et al.*, 1998; Kinoshita *et al.*, 1999; Lizard *et al.*, 2000). Cytochrome c was released in human monocytes, bovine endothelial cells and human endothelial cells (Lizard *et al.*, 2000). Membrane blebbing occurred in human arterial smooth muscle cells, lung fibroblasts and THP-1 monocytes (Björkerud and Björkerud, 1996). Bcl-2 or Bcl-x_L decreased in human coronary artery endothelial cells (Li *et*

al., 1998), human monocytes, bovine and human endothelial cells (Lizard *et al.*, 2000) and human vascular smooth muscle cells (Siow *et al.*, 1999). A Ca^{2+} rise was seen in cultured lymphoblastoid cells (Escargueil *et al.*, 1992) and inferred in neuronal cultures through use of a Ca^{2+} chelator (Keller *et al.*, 1999), and in Chinese hamster ovary cells through discovery of an apoptosis-resistant mutant unable to take up Ca^{2+} (Rusinol *et al.*, 2000).

Various components of oxLDL have been examined separately for their effects on apoptosis. 7β-hydroxycholesterol and 25-hydroxycholesterol caused apoptosis in U937 cells and HL60 cells (Yuan *et al.*, 2000), as did 7-ketocholesterol (Lizard *et al.*, 1998), which also induced apoptosis in bovine endothelial cells and human endothelial cells (Lizard *et al.*, 2000). 7-Ketocholesterol and 7β-hydroxycholesterol induced apoptosis in smooth muscle cells and endothelial cells (Nishio *et al.*, 1996; Lizard *et al.*, 1999). 2-Oxoaldehydes caused apoptosis in U937 cells and RAW 264.7 cells (Okado *et al.*, 1996). Mouse peritoneal macrophages and rabbit aortic smooth muscle cells were found not to undergo apoptosis with lysophosphatidylcholine (Nishio *et al.*, 1996; Niu *et al.*, 1996). *Tert*-butylhydroperoxide caused DNA single strand breaks in U937 cells (Brambilla *et al.*, 1998).

Aggregated LDL inhibited apoptosis in THP-1 cells and human peripheral blood monocytes stimulated by PMA (Kubo *et al.*, 1997). AgLDL induced the human homologue of E2 ubiquitin-conjugating enzyme, which may suppress apoptosis by poly-ubiquitination and degradation of proteins that induce apoptosis, including p53 (Kikuchi *et al.*, 2000). This form of LDL has also been shown to decrease levels of caspases-1 and -3 (Martinet and Kockx, 2001).

In some cases, oxLDL causes necrosis. This may be in a specific cell type, or due to a high concentration of oxLDL. Neuronal cultures and mesangial cells showed necrotic features with high concentrations of oxLDL (Sharma *et al.*, 1996; Keller *et al.*, 1999). OxLDL caused mainly necrosis in EBV-transformed B cells and lymphoblastoid cells, possibly because of increases in Bcl- x_L and Bcl-2 expression (Escargueil-Blanc *et al.*, 1997; Alcouffe *et al.*, 1999). The same effect was found if Bcl-2 was at high levels in HL60 cells

and lymphocytes (Meilhac *et al.*, 1999). Necrosis was also found in fibroblasts (Lizard *et al.*, 1999).

Often, necrosis is reported to follow apoptosis, if an incubation is carried out for long enough. A loss in membrane integrity will be found, indicated by trypan blue staining or the lactate dehydrogenase assay (Papassotiropoulos *et al.*, 1996; Escargueil-Blanc *et al.*, 1997). In this case, the necrosis is described as secondary, or post-apoptotic, necrosis. Secondary necrosis often occurs *in vitro* at the conclusion of the apoptotic process, due to the absence of other cells to engulf the apoptotic cells and clear the debris (Papassotiropoulos *et al.*, 1996). This was found in J774 cells and P388D_1 cells after 48 hours (Reid *et al.*, 1993A; Yuan *et al.*, 2000), rat dorsal root ganglion cells and THP-1 monocytes at 24 hours (Papassotiropoulos *et al.*, 1996; Vicca *et al.*, 2000), and in human endothelial cells at around 12 hours, the same time as changes were measured by the MTT assay (Escargueil-Blanc *et al.*, 1997).

Antioxidants have been able to reduce apoptosis caused by oxLDL. Superoxide scavengers decreased oxLDL-apoptosis in neuronal cultures (Keller *et al.*, 1999) and human coronary artery endothelial cells (Li *et al.*, 1998) and SOD and catalase inhibited the apoptosis of HUVECs (Galle *et al.*, 1999).

GSH, N-acetylcysteine and vitamin E could stop apoptosis in U937 cells with 7-ketocholesterol, reducing production of superoxide. Vitamin C and melatonin did not delay apoptosis, although superoxide production was prevented (Lizard *et al.*, 2000).

Three assays are used here to try to define the type of apoptosis that may be occurring in THP-1 and U937 monocyte cells. A fluorescent caspase assay examines the activity of caspase-3, the Annexin V/PI assay looks at the externalization of phosphatidylserine in the cell membrane and the cells' internalization of propidium iodide dye, and the Hoechst stain allows details of nuclear morphology to be visible. Together, the three features provide a broad view of apoptotic development over time and different parts of the process. Their relevance and the importance of testing for these particular characteristics are discussed later. The possibility of 78NP influencing these features of the apoptotic process is also investigated.

RESULTS AND DISCUSSION

A. ACTIVATION OF CASPASE ENZYMES

Caspases are possibly the best biochemical marker of apoptosis, since there is such limited evidence that they appear in necrosis (Leist and Nicotera, 1997). Their presence alone is often considered proof of apoptosis. This reliance may be problematic, as, for example, an immunoassay may simply pick up the inactive zymogen, showing the cell is capable of apoptosis, but not that apoptosis is occurring (Bennett and Boyle, 1998). There are also some instances of apoptosis in which caspase activation does not appear.

Caspase-3 is an executioner caspase, carrying out the protein-cleavage processes of apoptosis, rather than merely forming part of the signalling cascade. Its presence would therefore be a reliable indicator of the occurrence of caspase-mediated apoptosis in the cells at the time of freezing. Much of the DEVD-AMC activity is due to caspase-3, although other effector caspases may potentially have a role (Stridh *et al.*, 1998; Hampton *et al.*, 2002B). 3% Ethanol was used as a positive control in these experiments, as it had already been shown to induce caspase activity in THP-1 cells (Brown *et al.*, 1996).

A timecourse looking at oxLDL-induced caspase activity was performed on each cell type over 48 hours, using four different oxLDL concentrations.

Once oxLDL was added to THP-1 cells, the caspase activity began to increase steadily (Figure 62). The increase started earlier the higher the concentration of oxLDL. The extent of the rise was less for the higher concentrations: it peaked and decreased again, and in the case of 3.0mg/mL oxLDL, was back down to control levels by 48 hours. In keeping with this pattern, the 1.5mg/mL oxLDL sample was the next to begin increasing, which it did marginally at 6 hours, taking off at 12 hours, then peaking and beginning to fall again by 24 hours. The 0.5mg/mL oxLDL sample began its rise at 12 hours, and was still increasing by the end of the timecourse, as was the 0.2mg/mL oxLDL sample, which showed increasing caspase activity from 24 hours.

If caspase activity is used as a definition of apoptosis, all the concentrations of oxLDL used here induce apoptosis in THP-1 cells, albeit at different rates and to varying extents. The drop in caspases seen to occur with the higher concentrations of oxLDL, which will probably also occur with the lower concentrations, in time, may be due to the onset of secondary necrosis. This view is supported by the data gathered from the studies of phosphatidylserine exposure and nuclear morphology in the following sections of this chapter.

The increase in caspases appears to be an early sign of cell death, found before any changes were detected by the two cell viability assays used in other chapters. This reiterates the fact that the trypan blue and MTT assays measure the later stages of cell death.

The addition of increasing concentrations of 78NP to THP-1 cells incubated with 1.5mg/mL oxLDL did not significantly affect the level of caspase activation (Figure 63), much as it could not influence the level of cell viability in this cell type (Figure 25).

Previous studies reported that 78NP and neopterin caused apoptosis. However, 200μM 78NP and 200μM neopterin did not cause an increase in caspase activity alone. Pterin levels said to have this effect are usually in the millimolar range (Enzinger *et al.*, 2001), but 200μM 78NP and 200μM neopterin were described as enhancing IFNγ and TNFα-mediated apoptosis (Schobersberger *et al.*, 1996).

U937 cell control samples' caspase activity rose slightly over time, and cells incubated with 0.2mg/mL oxLDL were not significantly different (Figure 64). The remaining oxLDL concentrations caused a drop in caspase activity which began earlier and happened at a faster rate the higher the concentration of oxLDL. This drop represented an almost total obliteration of any caspase activity found.

The level of caspase activity found over 48 hours with oxLDL was markedly lower in U937 cells than in THP-1 cells. The highest value for the THP-1 cell timecourse was 58.07±1.05 pmol AMC/min/10^6cells, but for U937 cells it was only 2.67±0.19 pmol AMC/min/10^6cells. This highest level for the U937 cells was lower than that found for the THP-1 cells' control and was therefore practically non-existent. So while the trends for this

cell type are examined, it is worthwhile bearing in mind that the scale on which they occur is very small.

Continuing the trend of having an effect with U937 cells, but not with THP-1 cells, 78NP was able to alter the effect of the oxLDL on caspase activity. The four graphs in Figure 65 each represent one oxLDL concentration, and show the effect of 200μM 78NP on it over time. The concentrations are expressed separately for reasons of clarity. To make it easier to see both how the oxLDL has changed the caspase activity and how 78NP has influenced this, the information on the graphs is expressed relative to the control.

With 0.2mg/mL oxLDL, no real deviation from the control caspase levels was found, and the addition of 78NP did not alter this. With 0.5mg/mL oxLDL, the drop observed at 12 hours was countered by the 78NP, which caused a large rise above the control, resolving itself to control levels as the oxLDL-only sample leveled out. So once the oxLDL began to cause a decrease in the caspase activity, the 78NP compensated for this. At 24 hours, it was over-compensating, but only to the level of the control in the THP-1 cells, not an activation great enough to contribute to the development of apoptosis.

With 1.5mg/mL oxLDL, the drop in caspase activity came earlier, and 78NP was able to reduce the decrease until 24 hours, after which no further significant difference between the two was found. With 3.0mg/mL oxLDL, the trend was much the same as with 1.5mg/mL.

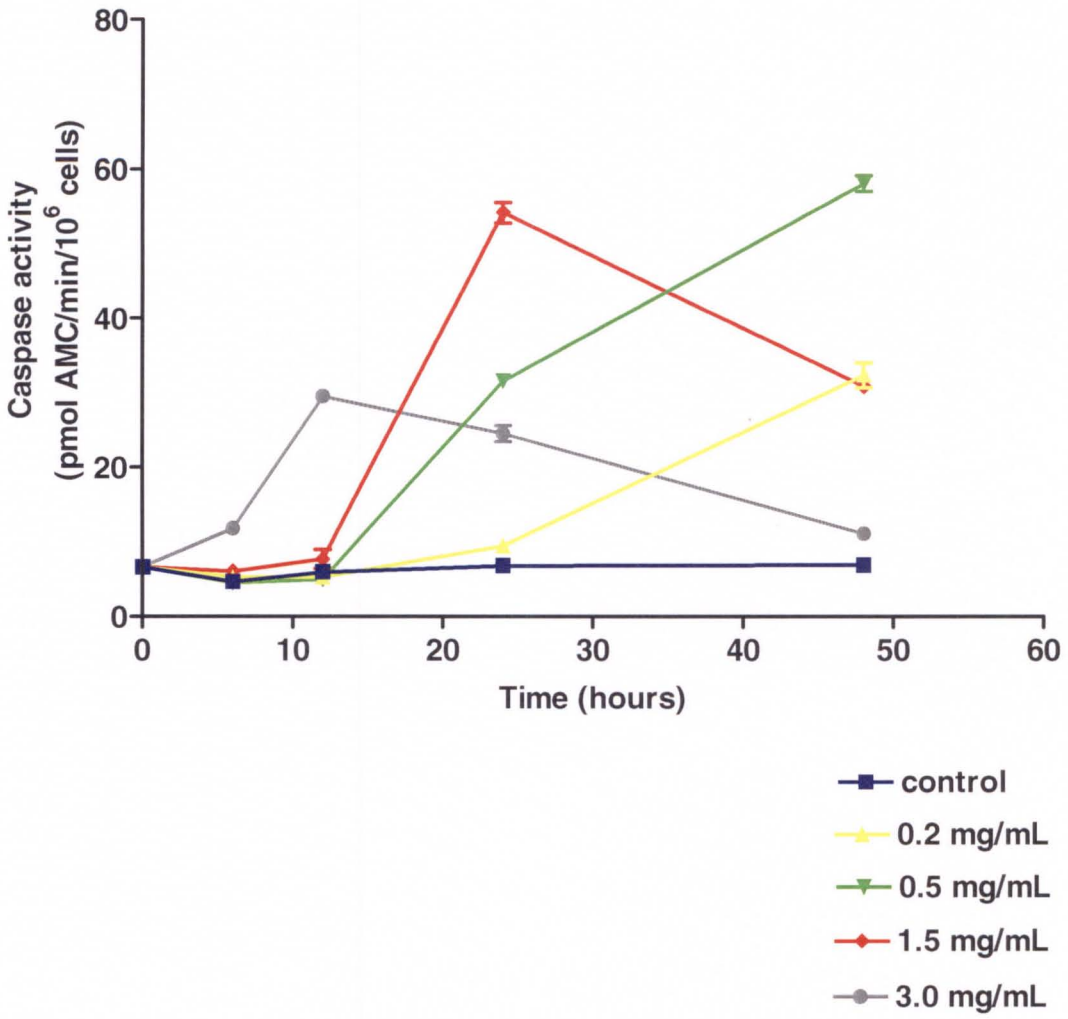

Figure 62: A timecourse study of the effect of increasing oxLDL concentrations on caspase activity in THP-1 cells. THP-1 cells (5 x 10^5cells/mL) were incubated with varying oxLDL concentrations for up to 48 hours in RPMI 1640. At certain times, cells were removed, washed and prepared for analysis by caspase assay. The results are expressed as the mean ± SD of triplicates.

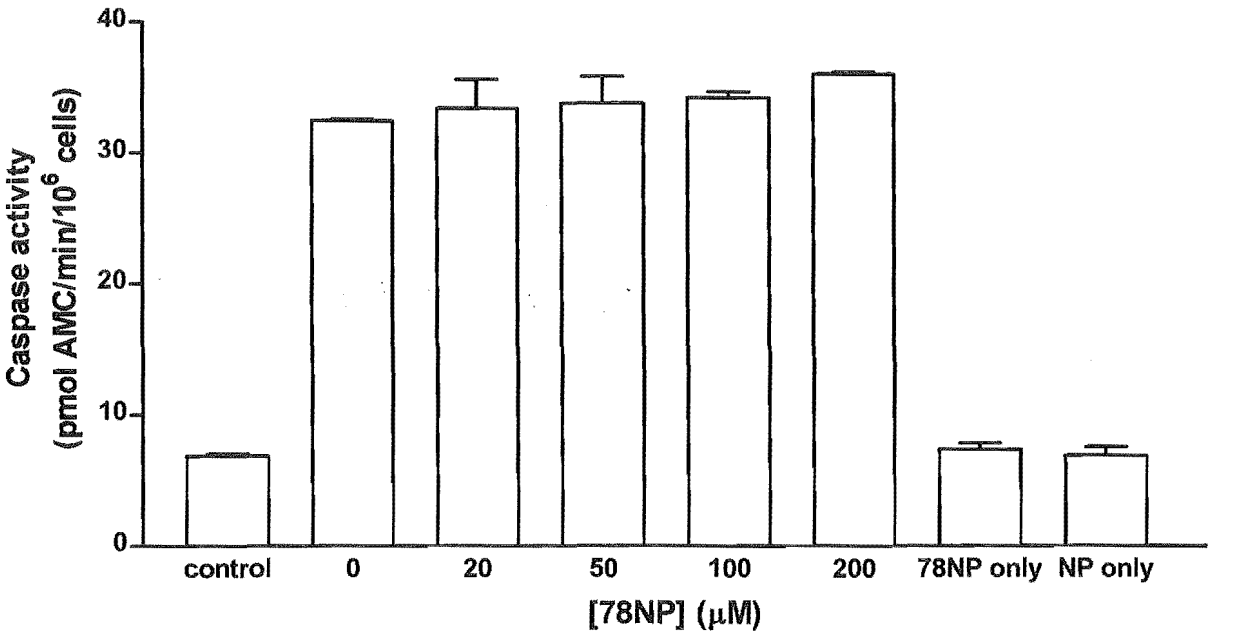

Figure 63: The effect of increasing concentrations of 78NP on oxLDL-induced caspase activity in THP-1 cells. THP-1 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL for 48 hours in RPMI 1640. The cells were then washed and prepared for analysis by caspase assay. Two controls, with 200μM 78NP or 200μM neopterin (NP), without oxLDL are included. The results are expressed as the mean ± SD of triplicates.

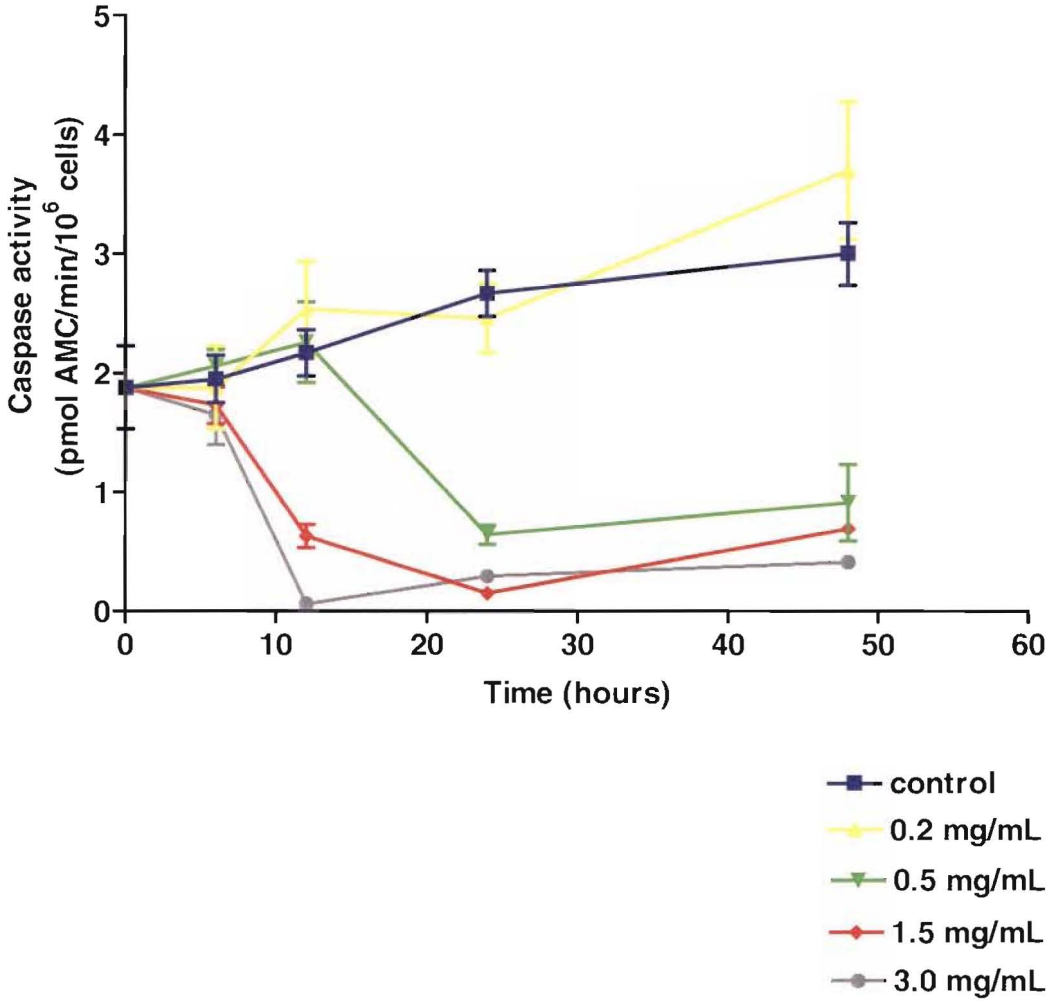

Figure 64: A timecourse study of the effect of increasing oxLDL concentrations on caspase activity in U937 cells. U937 cells (5 x 10^5cells/mL) were incubated with varying oxLDL concentrations for up to 48 hours in RPMI 1640. At certain times, cells were removed, washed and prepared for analysis by caspase assay. The results are expressed as the mean ± SD of triplicates.

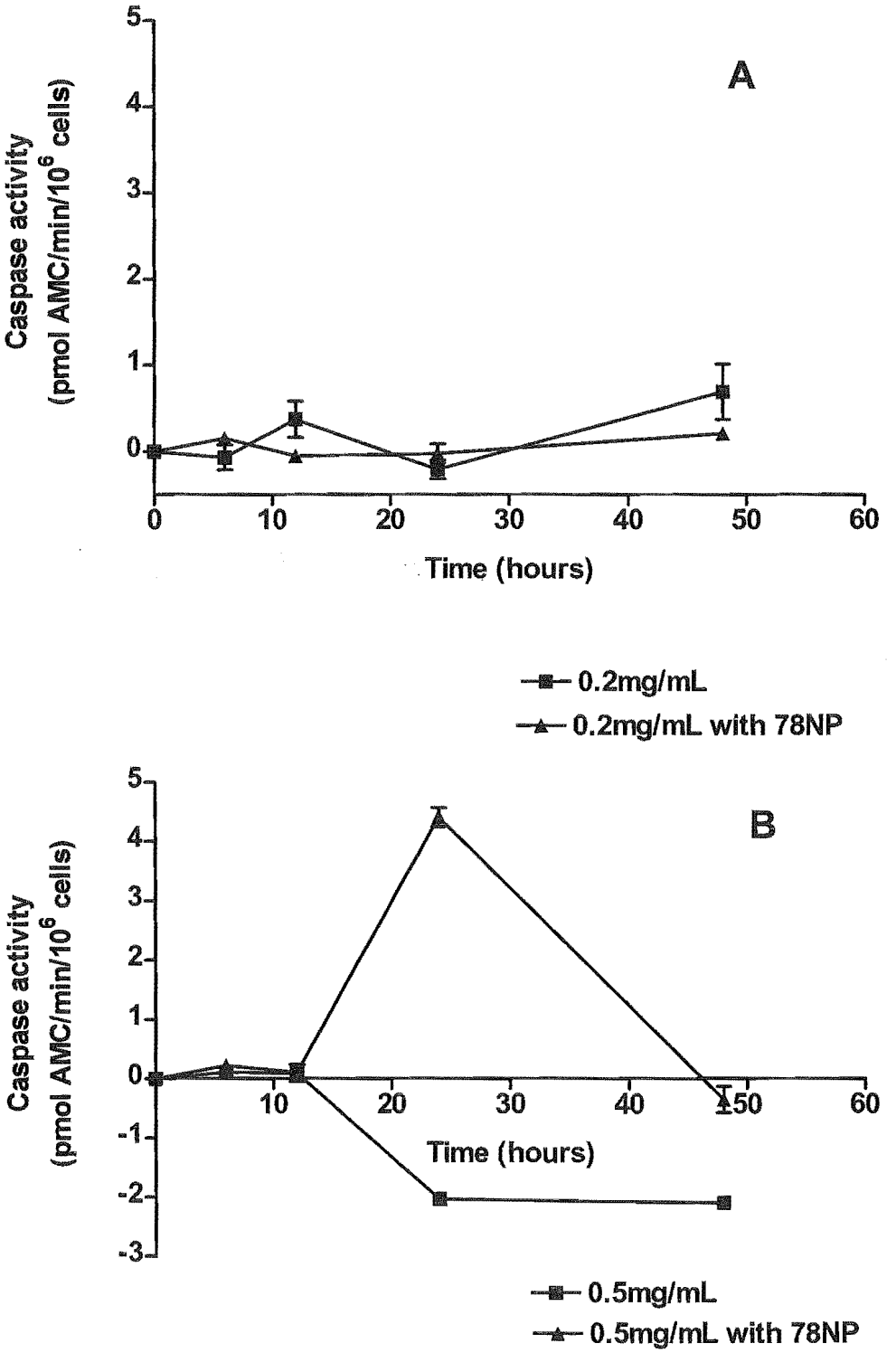

Figure 65: The effect of 78NP on oxLDL-induced caspase activity in U937 cells.
U937 cells (5 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL with or without 200μM 78NP for up to 48 hours in RPMI 1640. The cells were then washed and prepared for analysis by caspase assay. The results are expressed as the mean ± SD of triplicates. This Figure is continued on the next page.

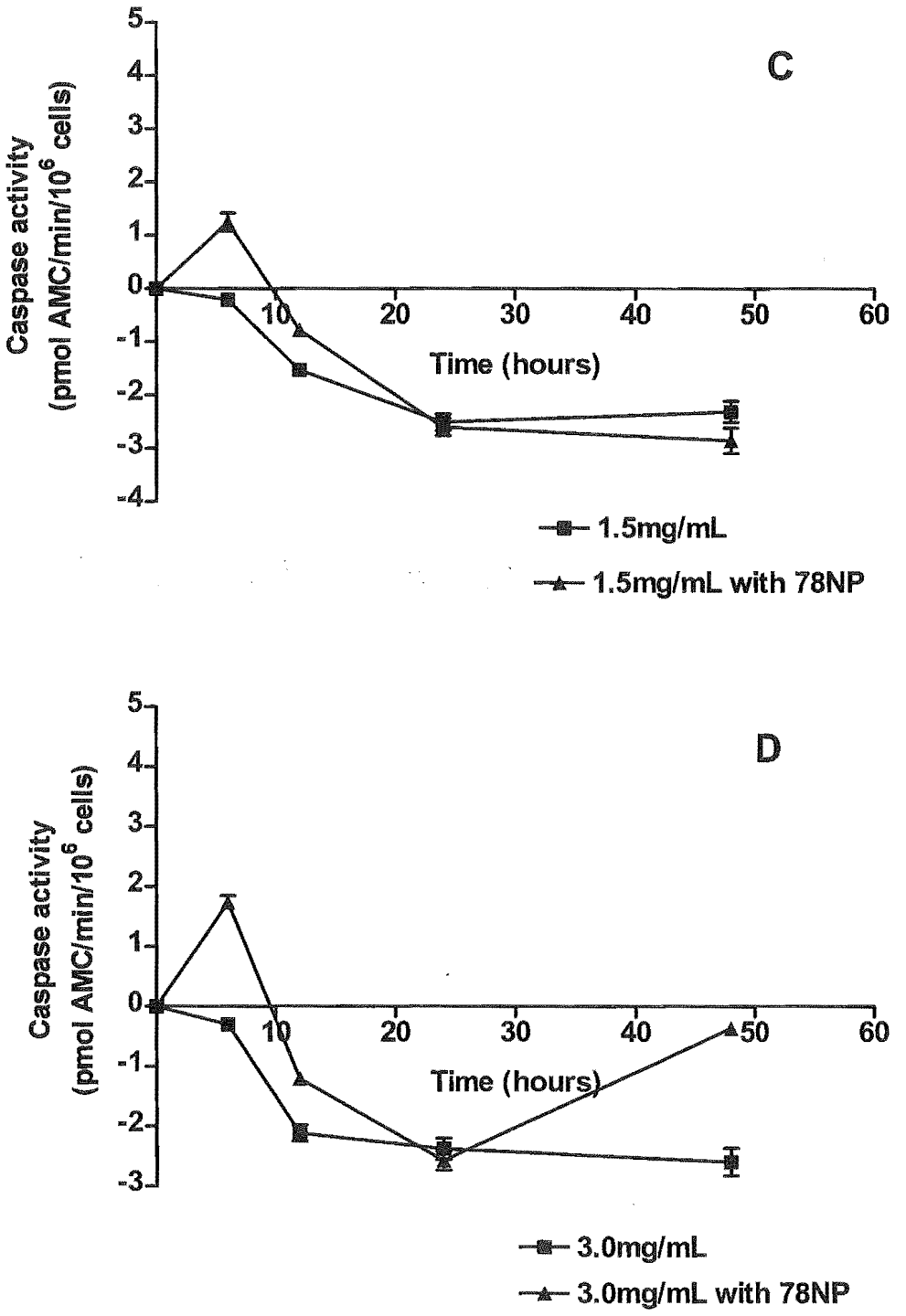

Figure 65: The effect of 78NP on oxLDL-induced caspase activity in U937 cells.
U937 cells (5 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL with or without 200μM 78NP for up to 48 hours in RPMI 1640. The cells were then washed and prepared for analysis by caspase assay. The results are expressed as the mean ± SD of triplicates.

The activation of caspases as part of oxLDL-mediated cell death is another difference between THP-1 and U937 cells in response to oxLDL.

The lack of caspase activity in U937 cells may be a feature specific to oxLDL, as there are several reports in the literature of caspase activation in U937 cells. Some were tested using fluorescent substrates as was carried out here, and in others, caspase activity was inferred from the ability of caspase inhibitors to stop apoptosis. Caspase-dependent apoptosis was found in U937 cells incubated with cadmium, heat, X-rays (Galán *et al.*, 2001), FasL, TNFα, and UV irradiation (Varela *et al.*, 2001; Exner *et al.*, 2002). U937 cells incubated with the plant lectin ricin were found to have caspase-3 and -6 activity (Sadakata *et al.*, 2000), if incubated with camptothecin (topoisomerase I inhibitor) caspases-1 and -3 were active (Yu *et al.*, 2000), with etoposide (topoisomerase II inhibitor) there was caspase-8 and -3 activity (Lizard *et al.*, 1998) and with arsenic trioxide, caspase-3 activation was achieved (Jing *et al.*, 1999; Park, *et al.*, 2001).

U937 cells may only use caspases in apoptosis in some situations. In U937 cells irradiated with X-rays, 5 and 20 Gy induced different forms of apoptosis. The high dose caused death before division and the low dose caused death only in postmitotic cells. Caspase inhibitors were only effective for the higher dose in the premitotic cells (Shinomiya *et al.*, 2000).

In an interesting case, U937 cells activated caspases-8 and -3 in response to cladribrine. In spite of this, a general caspase inhibitor had only a very small effect on morphology development, mitochondrial potential loss and phosphatidylserine exposure. The caspases may not play an early role in apoptosis in this situation, instead being activated by cytochrome c release and enhancing apoptosis (Marzo *et al.*, 2001). The low levels of active caspases found in U937 cells with oxLDL here may similarly represent a different system for the carrying out of apoptosis, or may show that the U937 cells are not undergoing apoptosis.

In U937 cells treated with 7-ketocholesterol, a component of oxLDL, procaspase-8 cleavage was found, but there was only a slight cleavage of procaspase-3 (Lizard *et al.*, 1998). Caspase-3 was thought to have only a minor role in this apoptosis. The alternative was considered to be a rapid progression to secondary necrosis, so that caspase-3 was difficult to

detect. Here, caspase-3 activity and variations in its levels were in fact detected, making this an unlikely reason for its apparent absence.

There are no examples in the literature comparing the different apoptotic pathways of U937 and THP-1 cells which might help to account for the differing use of caspases.

Recalling the extra sensitivity of the reduced thiol components of U937 cells to oxLDL (Figure 29), it was considered that this might be linked to the lack of caspase activity found under the same conditions. Caspase enzymes have an essential catalytic site cysteine residue. It is sensitive to the redox state of the cell, and responds to alterations in the glutathione ratio (Boggs *et al.*, 1998; Vissers *et al.*, 1999). The cysteine residue's oxidation by oxLDL would therefore render caspase enzymes inactive. In U937 cells incubated with 7-ketocholesterol, levels of caspase-3 declined through nitrosylation of its p17 subunit (Lizard *et al.*, 1998). One of the reasons a higher oxidative stress level induces necrosis is that the cell cannot maintain a reducing environment and repair oxidised caspases (Hampton and Orrenius, 1997). The 78NP effect found here on caspases could preserve the reduced thiol residues in the enzyme.

Figure 66 summarises the effect of 1.5mg/mL oxLDL on viability, thiol levels and caspase activity in U937 cells, and enables comparison of the timing and magnitude of change of these three parameters. Cell viability initially rose, then fell gradually. The decrease in caspase activity and reduced thiols mirrored each other more closely. They fell slowly up to 6 hours, then dipped sharply to 12 hours, remaining relatively static thereafter. This provides support for the idea of a relationship between thiol and caspase activity loss in U937 cells.

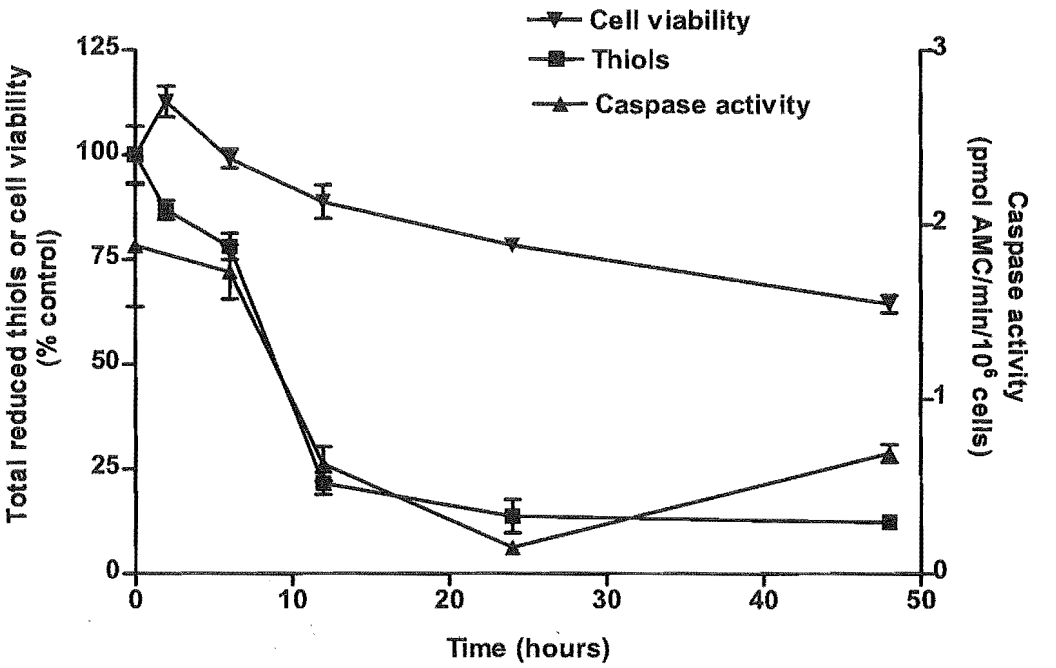

Figure 66: Comparison of loss of cell viability, reduced thiols and caspase activity in U937 cells with 1.5mg/mL oxLDL over 48 hours. U937 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL for up to 48 hours in RPMI 1640. Cells were removed at various timepoints and analysed by MTT assay, DTNB assay or caspase assay. The results are expressed as the mean ± SD of triplicates.

Examples of this type of situation have been found previously. Hydrogen peroxide impaired caspase activity, both in intact cells and cell lysates. If added to Jurkat T cells 30-90 minutes after Fas, the hydrogen peroxide directly inactivated the caspases, also delaying blebbing and fragmentation. The effect was temporary, and after the hydrogen peroxide was consumed, apoptosis proceeded (Hampton and Orrenius, 1997). The same was seen in macrophages after apoptosis initiation by NO or serum withdrawal. Caspases-8 and -3 were inhibited, and this was prevented and reversed by dithiothreitol (Borutaite and Brown, 2001).

When Jurkat T cell lysates were incubated with DEVD-AMC, and hydrogen peroxide was added, immediate inhibition of caspases was seen (IC_{50} 7μM). After removal of the hydrogen peroxide, DTT could regenerate the caspase activity, but only very slowly. If hydrogen peroxide was added before the substrate, the IC_{50} was 310μM. It was suggested the substrate might induce conformational changes in the enzyme, making it more reactive to the hydrogen peroxide (Hampton *et al.*, 2002B).

Protein peroxides added to Jurkat cell lysates after apoptotic induction with anti-Fas antibody also reduced caspase activity. The activity could be partially restored with DTT, showing that again the effect was thiol-dependent. Tryptophan-derived peroxides were the most effective, requiring only physiological concentrations, however whole proteins had no effect. The inhibition was at a thirtieth of the concentration of hydrogen peroxide required. The presence of substrate impaired reactivity, the opposite effect found from that with hydrogen peroxide, showing the peroxides were targeting the active site (Hampton *et al.*, 2002A).

Other substances can inactivate caspases through reaction with thiols. NO can inhibit caspases through nitrosylation of the active site thiol by a cGMP-independent mechanism (Boggs *et al.*, 1998). Selenium repressed caspase-3 activity in human embryonic kidney cell lysates irradiated with UVB, as well as purified recombinant caspase-3 activity. DTT could reverse the effect, indicating it was through oxidation of the cysteine residue (Park *et al.*, 2000). Zn^{2+}, which reacts with thiols, also inhibited caspases (Stennicke and Salvesen, 1997). Thiol-alkylating agents such as iodoacetamide or *N*-ethylmaleimide could block caspase activation in U937 cells incubated with the plant lectin ricin (Sadakata *et al.*, 2000) and actinomycin D (Mohr *et al.*, 1997).

In a mimic of the hydrogen and protein peroxide studies, the experiment represented in Figure 67 was designed to test whether oxLDL could have a disabling effect on caspase enzymes. The earlier experiments outlined above on caspases and reduced thiols in U937 cells suggested this might be the case.

The study was conducted with cell lysates over a very short time. A more pronounced effect on caspases might be expected over a greater timeframe. U937 and THP-1 cells were incubated with 6% or 3% ethanol respectively for 6 hours to activate caspase enzymes. The cells were then washed with PBS and assayed for caspase activity. After 150 seconds, 0.5mg of oxLDL was added to the cuvette and the rate of substrate cleavage every hundred seconds between 400 and 1000 seconds recorded. The volume added amounted to 24µl. An addition of 8µl (0.167mg) was also tested for comparison, but no effect was found. The same volume of PBS was added for control samples. The results are expressed as % change in substrate cleavage rate during this time, found by comparing the rates for hundred second intervals

between 400-500 and 900-1000 seconds. No disproportionate immediate drop occurred after oxLDL addition, making this comparison valid.

The rate of fluorescent substrate cleavage remained quite constant in THP-1 cells with ethanol only. A lowering of approximately another 10% was found when oxLDL was added, contrary to the caspase activity heightening effect oxLDL has when added to whole THP-1 cells. However, since this concentration amounts to 5.0mg/mL (0.5 mg added to 100μL), this would not be so surprising over time. It is interesting that it had such an immediate effect. The 8μL addition, a concentration close to the 1.5mg/mL oxLDL concentration which caused caspase activation over 48 hours, did not affect activation over this very short amount of time. If incubated with cell lysate for longer before analysis, it might have had an effect.

The difference in caspase activity with and without oxLDL was amplified in U937 cells. The overall caspase levels were still much smaller than in the THP-1 cells, as was found earlier. In U937 cells incubated only with ethanol, levels were found to increase around 25% over the 600 seconds, perhaps as more procaspase-3 was cleaved. Since the enzyme levels are lower, substrate availability is not a problem. Once oxLDL was also added, the levels dropped dramatically. The difference between the increase without oxLDL and the decrease with it is around 65%, a loss of over half the caspase activity.

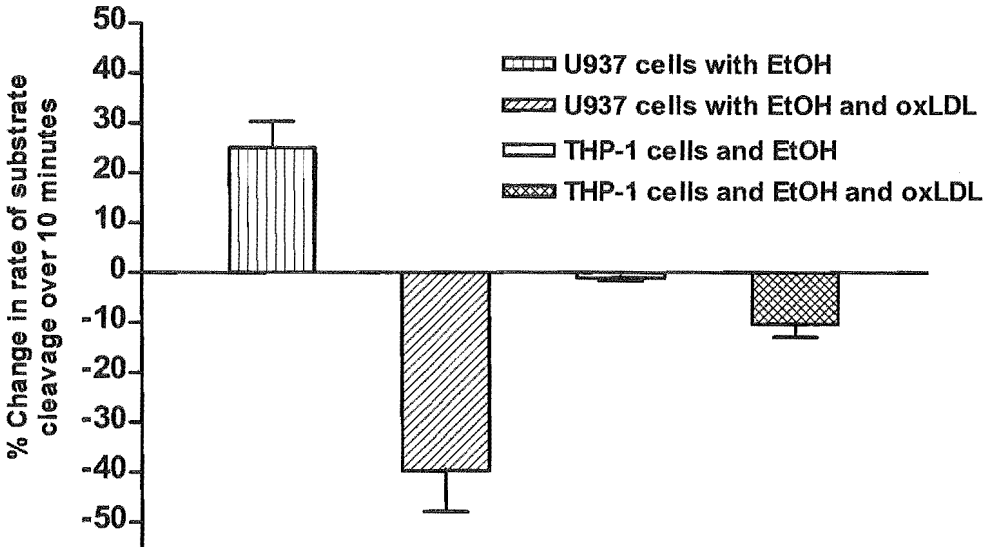

Figure 67: Effect on caspase activity of the addition of oxLDL. U937 cells and THP-1 cells at 5 x 10^5cells/mL were incubated with EtOH (6% and 3% respectively) for 6 hours in RPMI 1640 to activate caspase enzymes. The cells were washed, lysed and assayed for the caspase activity. After 150 seconds, 0.5mg oxLDL was added. Rate of substrate cleavage was recorded between 400 and 1000 seconds. The results are expressed as the mean ± SD of triplicates.

OxLDL appeared to oxidise the thiol of caspase enzymes in U937 cells to a greater extent than in THP-1 cells. The data does not differentiate between a direct and indirect effect. U937 cells may have a different form of intracellular oxidative stress, or be more sensitive to oxidative stress, or respond differently to oxLDL, creating an intracellular oxidative stress, which THP-1 cells do not experience (see discussion in Chapter 4). The appearance of a difference in oxLDL effect on caspases in cell lysates suggests that the varying effects of oxLDL on THP-1 and U937 thiol oxidation (Figures 28 and 29) was not due to a difference in access to the cell. Rather, the distinction related to intracellular components.

An increase in oxidative processes is found in human monocytes, bovine endothelial cells and human endothelial cells with oxLDL (Lizard *et al.*, 2000). This might include production of hydrogen peroxide, superoxide and hydroxyl radicals, as shown in endothelial cells incubated with oxLDL (Dimmeler *et al.*, 1997).

The tendency for the reactive site thiol to become oxidised will be increased by the loss of the cell's total reduced thiols, as occurred in U937 cells with oxLDL, but not in THP-1 cells. This would act as an indirect form of oxidative stress, passively allowing oxidation, without the need generate more radicals (Ghibelli *et al.*, 1998). It is less damaging than generating non-specific oxidants (Van den Dobbelsteen *et al.*, 1996). With a lower glutathione level a cell has a decreased ability to resist oxidation or scavenge reactive oxygen species (Buttke and Sandstrom, 1995). GSH depletion with L-buthionine-[S,R]-sulfoximine has been shown to potentiate oxLDL cytotoxicity (Bustamente *et al.*, 1995; Galán *et al.*, 2001).

A lowering of thiols is necessary in many cases for apoptosis to take place. Apoptosis may still be caspase-dependent, or if the thiols are completely depleted, the cells might then, as U937 cells do here, have a mechanism other than caspases for carrying out apoptosis. In U937 cells incubated with 7-ketocholesterol, GSH loss happened early on. Adding more GSH or N-acetylcysteine impaired apoptosis. This could also prevent etoposide-induced apoptosis, but not cycloheximide apoptosis. GSH was found to strongly inhibit early signs of apoptosis such as caspase-8 and cytochrome c release, but only delay DNA fragmentation and not affect caspase-3, the later signs (Lizard *et al.*, 1998). The fact that GSH was not effective in all situations suggests that although U937 cells may often lose their reduced thiols as part of apoptosis, the loss is also apoptotic mediator-specific.

The loss of glutathione may itself play a central role in apoptosis. For example, it may control mitochondrial transmembrane potential, since the permeability transition pore is a multi-ion thiol sensitive channel. This will have the effect of regulating the release of mitochondrial molecules such as AIF and cytochrome c. It can also regulate neutral Mg^{2+}-dependent sphingomyelinase, which makes ceramide (Lizard *et al.*, 1998; Liu *et al.*, 2001). In U937 cells incubated with 7-ketocholesterol, loss of endogenous GSH favoured apoptotic features such as decreased mitochondrial membrane potential and cytochrome c release, supporting the idea of a thiol-sensitive channel on mitochondria (Lizard *et al.*, 1998).

In some situations, if GSH remains, necrosis may occur instead of apoptosis (Liu *et al.*, 2001). The inhibition of GSH extrusion in U937 and hepatoma cells with puromycin and etoposide prevented apoptosis. The reduced form of GSH was found extracellularly (Ghibelli

et al., 1998). The same was observed in Jurkat cells with anti-Fas antibody (Van den Dobbelsteen *et al.*, 1996).

The caspase behaviour found in the THP-1 cells is much more typical of conventional apoptotic behaviour than that of the U937 cells. We cannot tell from this assay whether apoptosis is occurring in U937 cells. They may undergo necrosis with oxLDL, or a more unusual caspase-independent form of apoptosis, possibly as a result of the critical cysteine residue of the caspase enzymes becoming oxidised. Other ways of measuring and defining apoptosis must also be explored.

B. PHOSPHATIDYLSERINE EXPOSURE

Another feature of the apoptotic process is the flipping of phosphatidylserine in the cell membrane so that it is exposed to the outside of the cell. There it functions as part of the recognition process for phagocytes, which clear away apoptotic cells before they reach the pro-inflammatory stage of secondary necrosis (Yu *et al.*, 2000).

Phosphatidylserine exposure has been described as a strictly caspase-dependent process (Borner and Monney, 1999), but it is now known that it may be regulated by caspase-dependent or -independent pathways (Yu *et al.*, 2000). Experiments with stimulated neutrophils have suggested that the flipping of phosphatidylserine might instead depend on oxidative stress (Fadeel *et al.*, 1998; Hampton *et al.*, 2002C).

No indication of the process' regulation in THP-1 cells has been found, but in U937 cells exposed to cladribrine, in which caspases-8 and -3 were active, a general caspase inhibitor had only a very small effect on phosphatidylserine exposure. A non-caspase factor must also contribute (Marzo *et al.*, 2001).

Phosphatidylserine exposure as a result of incubation with oxLDL has been found in Jurkat T cells (Alcouffe *et al.*, 1999), HUVECs (Galle *et al.*, 1999), THP-1 monocytes (Vicca *et al.*, 2000) and human vascular smooth muscle cells (Siow *et al.*, 1999).

Annexin V is a Ca^{2+}-dependent protein that preferentially binds to phosphatidylserine, and propidium iodide (PI) is a dye (like trypan blue) that enters cells with damaged cell membranes and incorporates itself into the DNA. Since PI differentiates between early and

late apoptotic (necrotic) cells, the assay tends to under- rather than overestimate the level of apoptosis (Farber *et al.*, 1999).

Flow cytometry counts 10,000 cells individually according to fluorescence by Annexin V and propidium iodide. The results are presented on a two-dimensional dot plot, with PI on the y axis and Annexin V on the x axis. Each graph shows 2,000 cells. A cell with very little fluorescence of either sort, in the lower left of the plot, is described as being viable. Cells in the lower right quadrant of the graph, binding Annexin V but excluding PI, can be said to be apoptotic. Cells that are both PI dye- and Annexin V-positive are necrotic and may be undergoing secondary necrosis (Vermes *et al.*, 1995; Bacso *et al.*, 2000).

The aim was to assign cells to each of these populations. This was originally done using quadrants, but this method of dividing up the plot was found to be too rigid, and some cells seemed to be assigned to the wrong group. Differences in the position of each cell type's viable population were not accounted for. So instead, hand-drawn regions were used, the shape of which was more flexible. Each cell type had slightly different regions, based on where their control populations started out. The same regions were used for analysis of all results for that cell type.

As oxLDL concentration increased the main cell population was seen to shift up and to the right in both cell types, as shown in the selection of dot plots in Figures 68 and 69.

In THP-1 cells, the viable region was the highest bar in the control cells, and as the oxLDL concentration increased, it dropped away (Figure 70). There were hardly any cells remaining in this region after incubation with 1.5mg/mL or 3.0mg/mL oxLDL.

All changes between the control and 0.5mg/mL involved THP-1 cells moving from the viable to apoptotic populations, displaying phosphatidylserine on the cell surface. Incubation with oxLDL at 0.5mg/mL resulted in the largest apoptotic sample, but apoptosis then decreased with increasing oxLDL concentration, as primary or secondary necrosis increased to include most of the cells by 1.5mg/mL oxLDL. There is no significant difference between the results for 1.5mg/mL and 3.0mg/mL oxLDL.

The THP-1 cells moved from being 78% viable, to 61% apoptotic, to 80% necrotic by 1.5mg/mL oxLDL. The oxLDL concentrations shown here have no midway point, where half

of the cells are apoptotic and half necrotic. This picture fits in well with the caspase activity levels at 48 hours for the different concentrations (Figure 62).

The necrosis would be primary if the cells moved directly from the viable region to the necrotic region, or secondary if they reached the necrotic region via the apoptotic region. This progression might occur faster for greater oxLDL concentrations, hence the higher number of cells in this region for the higher oxLDL concentrations. Secondary necrosis may be the most likely possibility, since at 48 hours, caspase activation had already peaked and dropped away in cells incubated with 1.5mg/mL and 3.0mg/mL oxLDL. This implies that cells had passed through apoptosis at this point.

78NP at 200μM was not found to influence phosphatidylserine exposure significantly on THP-1 cells with oxLDL ($p = 1$ for each of the three populations) (Figure 71). A concentration of oxLDL between the apoptotic concentration (0.5mg/mL) and necrotic concentration (1.5mg/mL) was used, in an attempt to find a point at which a similar proportion of apoptotic and necrotic cells were present, perhaps giving the 78NP a better chance of having an effect. However, 1.0mg/mL oxLDL presented a similar profile to 0.5mg/mL oxLDL.

The effect of oxLDL on phosphatidylserine exposure in U937 cells (Figure 72) was more gradual. The control cells had a similar proportion of viable cells to that found with the THP-1 cells. At 0.5mg/mL oxLDL, U937 cells had approximately the same amount of apoptotic cells as THP-1 cells did, but also a higher proportion of viable cells. By 1.5mg/mL oxLDL, the percentage of viable cells had decreased, although not as drastically as in THP-1 cells. The rest of the cells were split evenly ($p = 0.096$) between the apoptotic and necrotic regions. By 3.0mg/mL oxLDL the U937 cells had caught up with the THP-1 cells again: there were almost no viable cells, few apoptotic cells, and a vast majority of cells undergoing (secondary) necrosis.

Both 10μM and 200μM 78NP were able to delay the effects of 1.5mg/mL oxLDL on U937 cells (Figure 73). There was a significant decrease in the number of necrotic cells with 10μM 78NP ($p = 0.039$) and a very significant decrease for the 200μM concentration ($p = 0.001$). For 200μM 78NP, there appeared to be more viable cells, although this result was not significant ($p = 0.91$). There was no significant change in the number of apoptotic cells ($p = 0.06$ for 10μM and $p = 0.13$ for 200μM).

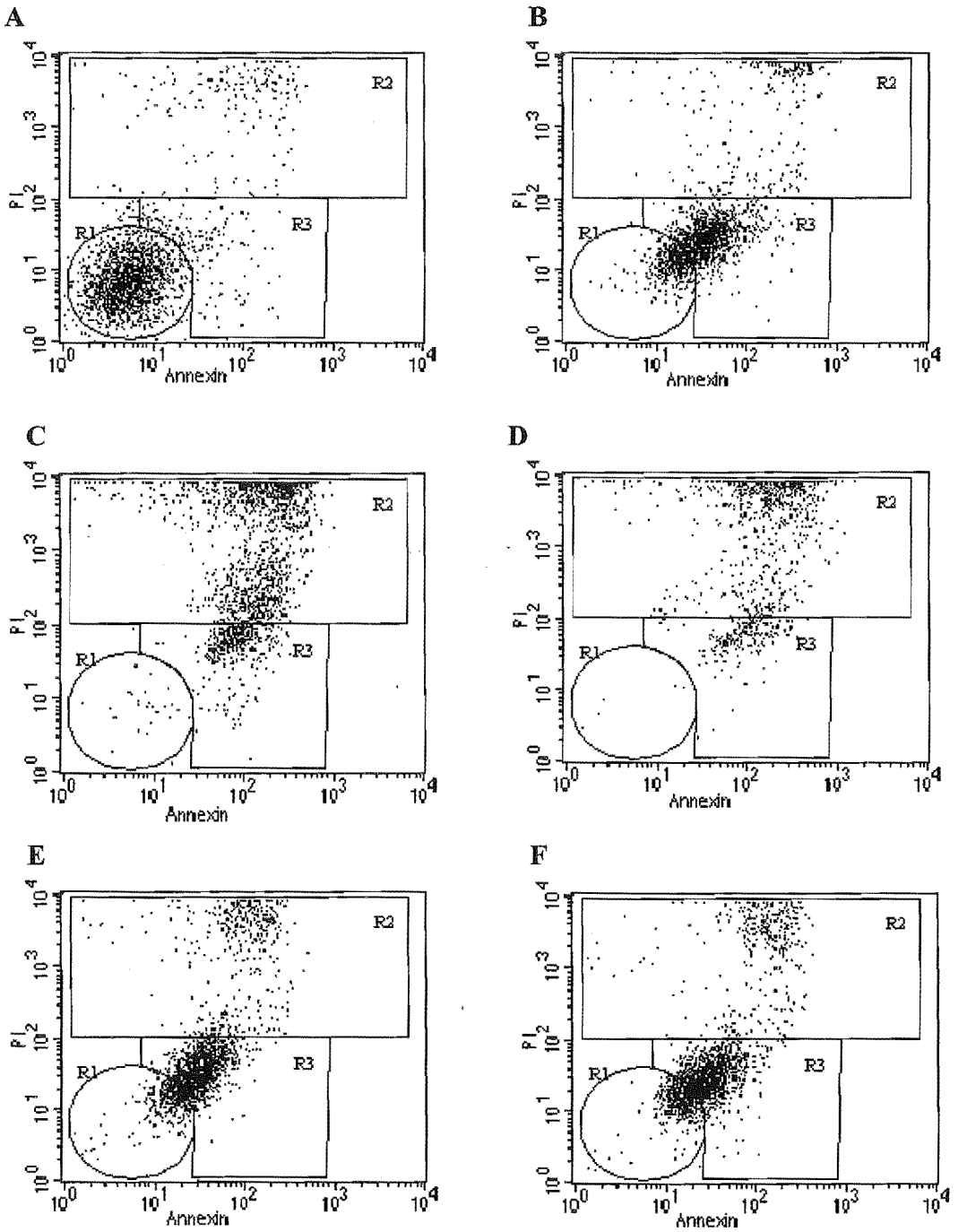

Figure 68: Flow cytometry dot plots showing the effect of oxLDL and 78NP on THP-1 cells.
THP-1 cells (5 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL or 78NP for 48 hours in RPMI 1640. The cells were washed and incubated with propidium iodide and annexin-V-FITC for 10 minutes before analysis by flow cytometry. Regions indicate approximate locations of cells that are viable (R1), undergoing apoptosis (R3) and in the process of (secondary) necrosis (R2). A shows control cells, B shows cells with 0.5mg/mL oxLDL, C shows cells with 1.5mg/mL oxLDL, D cells with 3.0mg/mL oxLDL, E cells with 1.0mg/mL oxLDL and F cells with 1.0 mg/mL oxLDL and 200μM 78NP.

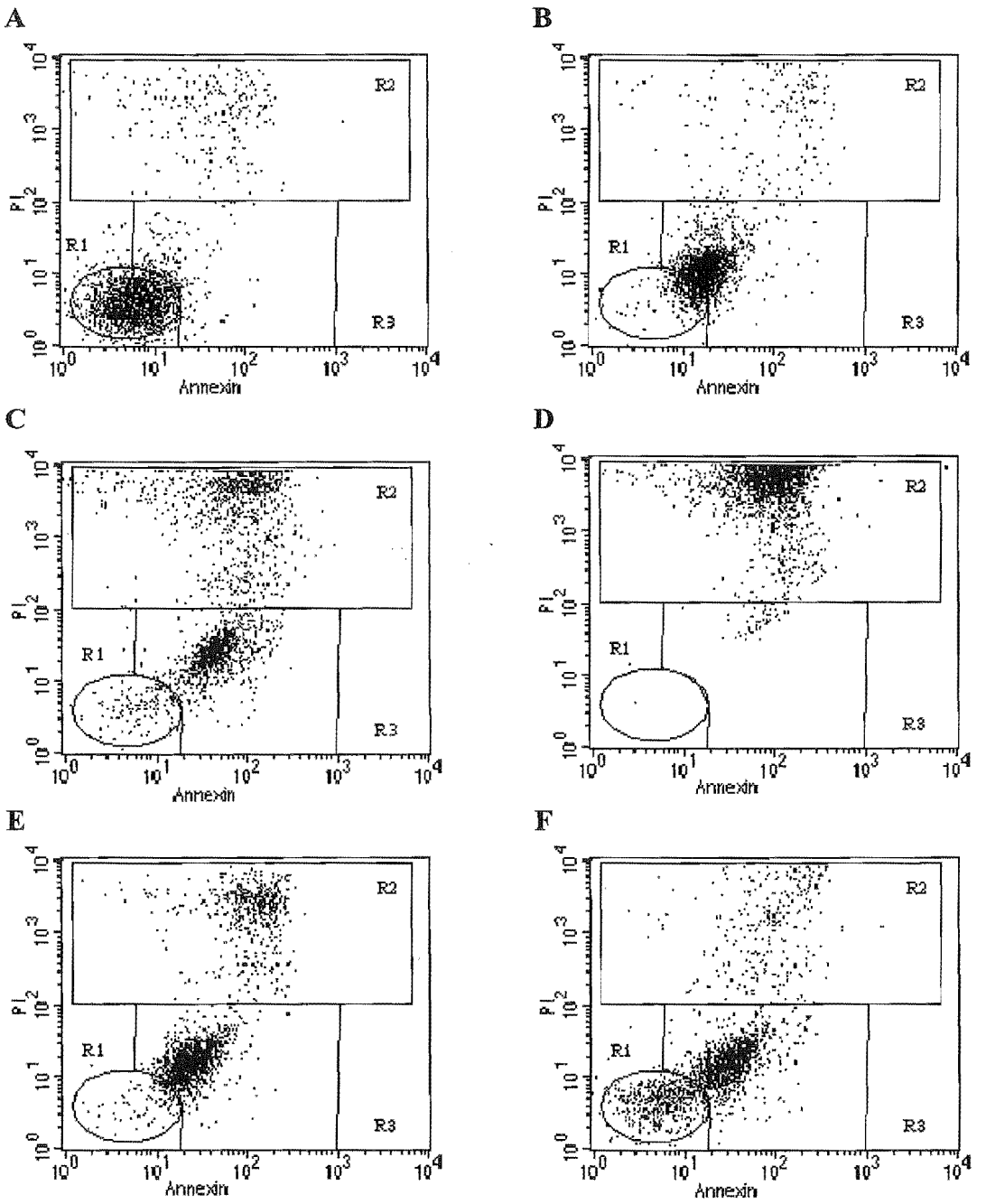

Figure 69: Flow cytometry dot plots showing the effect of oxLDL and 78NP on U937 cells.
U937 cells (5 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL or 78NP for 48 hours in RPMI 1640. The cells were washed and incubated with propidium iodide and annexin-V-FITC for 10 minutes before analysis by flow cytometry. Regions indicate approximate locations of cells that are viable (R1), undergoing apoptosis (R3) and in the process of (secondary) necrosis (R2). A shows control cells, B shows cells with 0.5mg/mL oxLDL, C shows cells with 1.5mg/mL oxLDL, D cells with 3.0mg/mL oxLDL, E cells with 1.5mg/mL oxLDL and 10μM 78NP and F cells with 1.5 mg/mL oxLDL and 200μM 78NP.

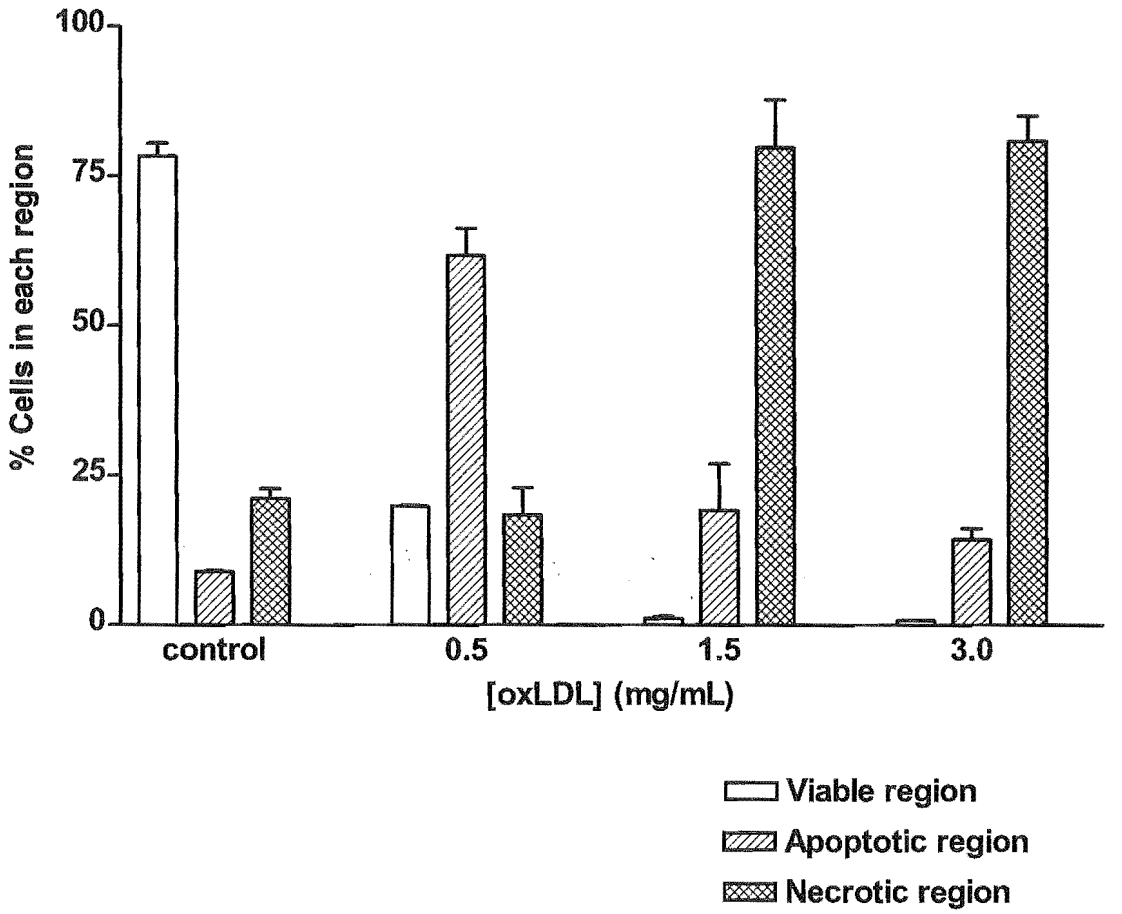

Figure 70: Effect of oxLDL on THP-1 cells' phosphatidylserine exposure and PI uptake.
THP-1 cells (5 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL for 48 hours in RPMI 1640. The cells were washed and incubated with propidium iodide and annexin-V-FITC for 10 minutes before analysis by flow cytometry. Regions were drawn on the dot plots to indicate approximate locations of cells that were viable, undergoing apoptosis and in the process of (secondary) necrosis. The number of cells in each area is expressed as the mean ± SD of triplicates.

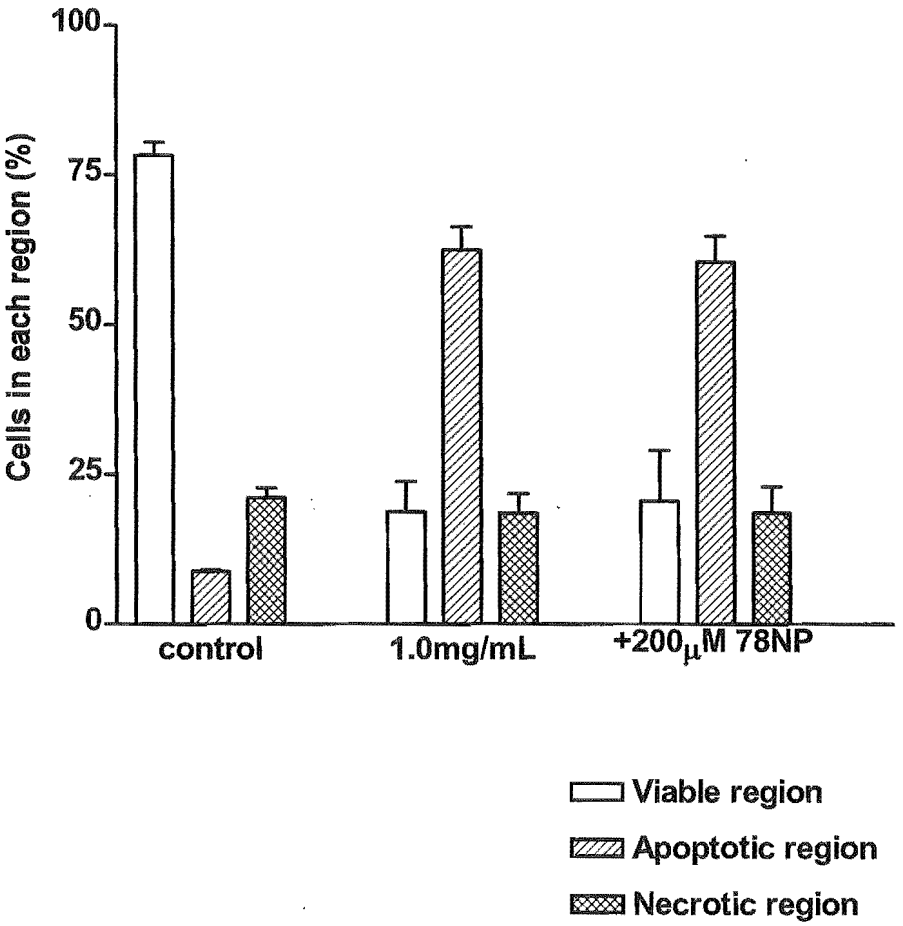

Figure 71: Effect of oxLDL and 78NP on THP-1 cells' phosphatidylserine exposure and PI uptake. THP-1 cells (5 x 10^5cells/mL) were incubated with 1.0mg/mL oxLDL with or without 200μM 78NP for 48 hours in RPMI 1640. The cells were washed and incubated with propidium iodide and annexin-V-FITC for 10 minutes before analysis by flow cytometry. Regions were drawn on the dot plots to indicate approximate locations of cells that were viable, undergoing apoptosis and in the process of (secondary) necrosis. The number of cells in each area is expressed as the mean ± SD of triplicates.

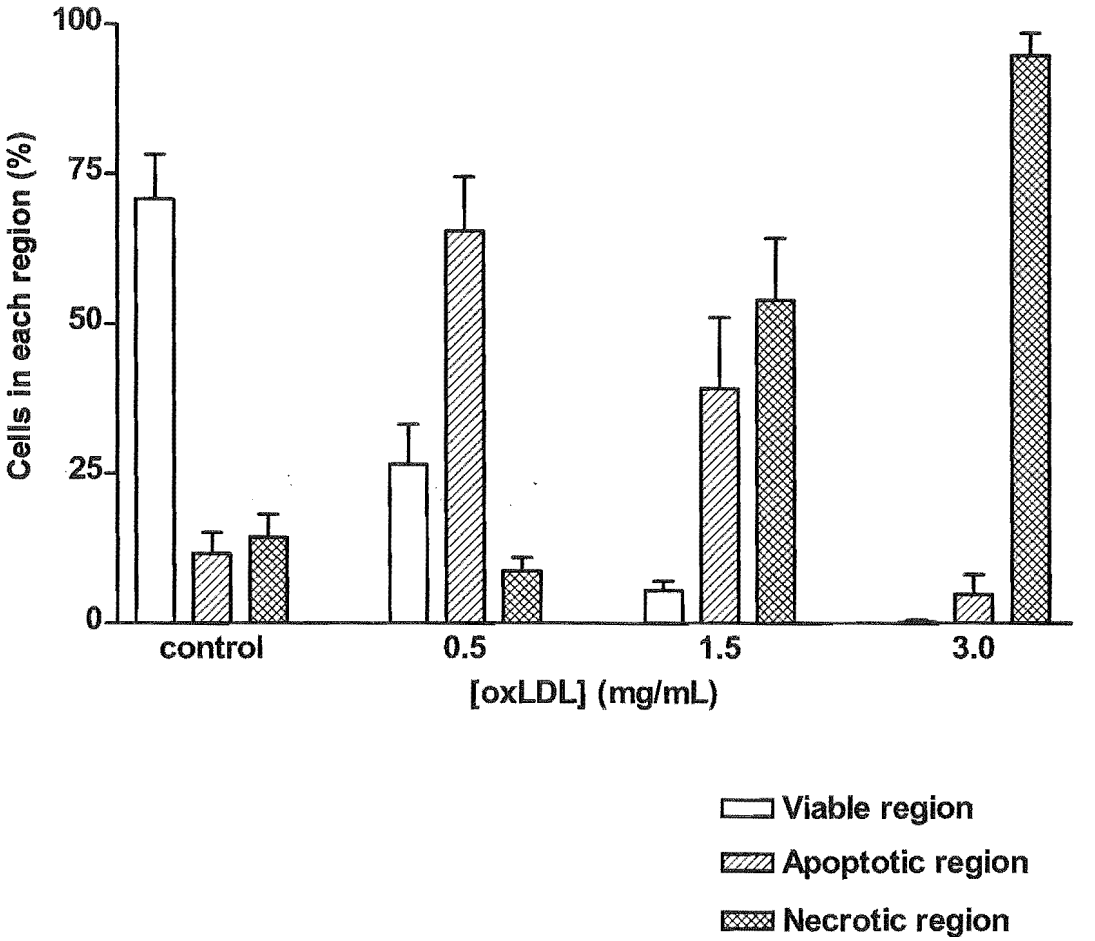

Figure 72: Effect of oxLDL on U937 cells' phosphatidylserine exposure and PI uptake.
U937 cells (5 x 10^5cells/mL) were incubated with increasing concentrations of oxLDL for 48 hours in RPMI 1640. The cells were washed and incubated with propidium iodide and annexin-V-FITC for 10 minutes before analysis by flow cytometry. Regions were drawn on the dot plots to indicate approximate locations of cells that were viable, undergoing apoptosis and in the process of (secondary) necrosis. The number of cells in each area is expressed as the mean ± SD of triplicates.

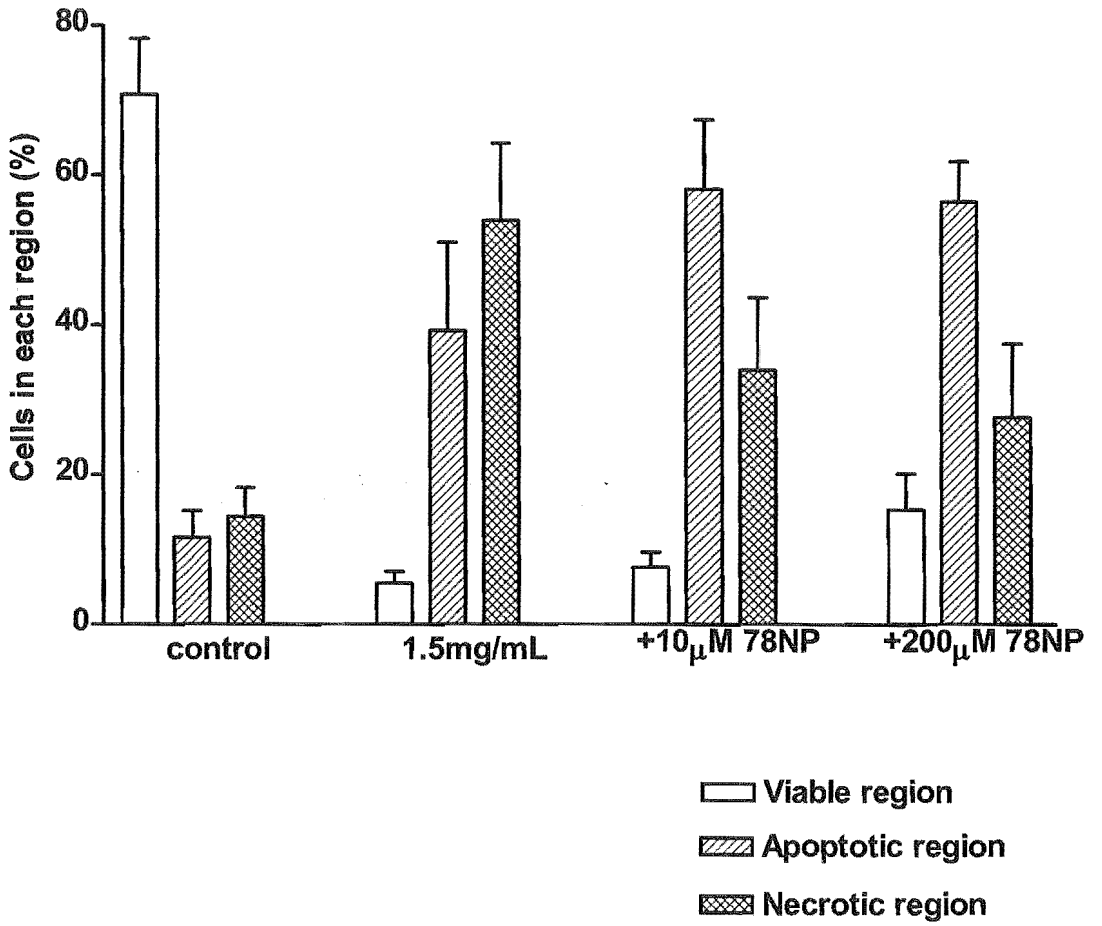

Figure 73: Effect of oxLDL and 78NP on U937 cells' phosphatidylserine exposure and PI uptake. U937 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL with or without 10μM or 200μM 78NP for 48 hours in RPMI 1640. The cells were washed and incubated with propidium iodide and annexin-V-FITC for 10 minutes before analysis by flow cytometry. Regions were drawn on the dot plots to indicate approximate locations of cells that were viable, undergoing apoptosis and in the process of (secondary) necrosis. The number of cells in each area is expressed as the mean ± SD of triplicates.

The 78NP effect is relatively small when compared to other 78NP protective effects in U937 cells, suggesting that its mechanism of action may be able to influence the phosphatidylserine exposure less efficiently than it would other parts of the cell death process. If 78NP acts as an antioxidant, as suggested by its thiol protective effect, the less significant effect on Annexin V binding may imply that the phosphatidylserine exposure process does not completely rely on the oxidative state of the cell. Alternatively, although 78NP may delay cell death, it may not directly affect phosphatidylserine exposure itself.

The lack of difference between the protective effects of the 78NP concentrations ($p = 0.999$) is in marked contrast to the effects of these concentrations on cell viability. If the maximum level of protection is provided by a much lower concentration, either the 78NP is much more efficient at this, or there is a maximum level of protection that is possible and already provided by the lower concentration.

By this assay's definition of apoptosis, apoptosis occurs in both cell types with oxLDL, as does (secondary) necrosis. The apoptotic process may occur more swiftly in THP-1 cells, as higher concentrations of oxLDL result in greater levels of (secondary) necrosis, a characteristic not picked up by the viability assays used earlier. This may relate to the THP-1 cells' use of caspases for the process, which are likely to be the most efficient way of completing apoptosis (Borner and Monney, 1999). The speed of conclusion of the death process may help to account for the lack of protection provided by 78NP, especially if its effect, as suggested here in the U937 cells, is to slow the process down. There is simply less of a window in which the 78NP can act. Perhaps the difference is also indicative of other dissimilarities in the carrying out of apoptosis, which 78NP cannot influence.

C. HOECHST STAIN

One of the later stages of the apoptotic cascade is the changes in the morphology of the cell, especially in the nucleus. Hoechst 33342 stains the nucleus of the cell, intercalating between the A-T base pairs of DNA, thereby making morphological changes of the nucleus visible (Schobersberger *et al.*, 1996). Changes include contraction and condensation of the nucleus, chromatin fragmentation, pyknosis (margination of the chromatin) and apoptotic body formation (Escargueil *et al.*, 1992; Okado *et al.*, 1996; Brunk *et al.*, 1997; Alcouffe *et al.*, 1999; Lizard *et al.*, 1999). Necrotic changes are also visible. These nuclei are described as swollen, diffuse and disintegrating (Lizard *et al.*, 1999).

This method is most suited to a qualitative analysis. The cells are examined until a feature of interest comes into view, which can then be photographed and described. Some authors attempt to categorise every cell by this method, as apoptotic, necrotic or neither. Cells can be difficult to assign to particular categories, so the decision seems likely to be subjective and less reliable.

THP-1 or U937 cells (5×10^5 cells/mL) were incubated with varying concentrations of oxLDL and 78NP in RPMI 1640 as before, but only for 24 hours, as it was found that many cells were too disintegrated after 48 hours to give a clear picture. At the end of the incubation, the cells were washed twice with PBS to remove the oxLDL, stained with Hoechst 33342 and mounted onto slides. The slides were examined using a fluorescence microscope at 40 or 100 times magnification.

Both cell types showed the traditional signs of apoptosis and necrosis in a similar way and to a similar extent. The only difference was in the effect of 78NP, which brought the cells closer to an untreated cell phenotype in U937 cells only.

Figures 74 and 75 show untreated THP-1 and U937 cells respectively. Both cell types have relatively rounded nuclei, with the nuclei distributed centrally in the cell.

Several of the features commonly found in cells undergoing apoptosis and stained with Hoechst 33342 are seen in the pictures of cells incubated with oxLDL. The condensation of the nucleus is represented by a shrinking in size and increase in stain brightness. Pyknosis

is seen by the nucleus moving to the edge of the cell. Fragmentation is also visible, as the nucleus breaks up into smaller pieces, while remaining in the cell. This is the beginning of the formation of apoptotic bodies. Good examples of each of these are marked in the figures with P for pyknosis, C for condensation and F for fragmentation, but there are other examples in each picture. Examples of necrotic features are indicated on the figures with S for swelling, DF for diffuse, DE for cell debris and DS for disintegrating cells.

The pictures of THP-1 and U937 cells incubated with 0.5mg/mL oxLDL for 24 hours show all the apoptotic features, and have no characteristics of necrosis (Figures 76 and 77). The main feature of Figure 77 is the brightness.

Figures 78-81 are cells incubated with 1.5mg/mL oxLDL. Figure 78 is of THP-1 cells, showing brightness and good fragmentation. Figure 79 features brightness, condensation and fragmentation in THP-1 cells. The very large cell appears to contain fragmentation in the manner of apoptosis, but the swelling is characteristic of necrosis, suggesting that, after 24 hours of incubation with this concentration of oxLDL, some cells have moved through apoptosis to secondary necrosis. This is an interesting finding as it suggests that condensation and fragmentation, followed by lysis, may be the mechanism of secondary necrosis. This has not been previously demonstrated.

Figure 80 shows two good examples of fragmentation and one of pyknosis in U937 cells. There are not many cells on the slide, suggesting some may already have undergone cell lysis. Figure 81 illustrates the beginnings of necrosis in U937 cells, in a similar way to Figure 79. Some cells appear diffuse, and seem to be disintegrating. Others are still undergoing apoptosis, as demonstrated by pyknosis and fragmentation.

Figures 82 and 83 were taken of cells incubated with 200μM 78NP as well as 1.5mg/mL oxLDL. The THP-1 picture (Figure 82) looks similar to the cells incubated only with oxLDL (Figure 79). There is fragmentation and swelling and diffuse cells. In the next figure, representing the U937 cells (Figure 83), a definite effect of 78NP can be seen. Care was taken to ensure the pictures represented what was present on the slides overall. The number of cells has markedly increased, suggesting death is slower. A few cells are still showing apoptotic features, but many look relatively normal, similar to the cells in the untreated population. Although the outcome of incubation with 78NP cannot be quantified here, it seems to be one of its more complete effects on apoptosis. This may relate to the fact

that these nuclear changes happen relatively late in the apoptotic process, so that, if delayed, they may be unlikely to have occurred by this 24 hour timepoint.

The last three figures show cells incubated with 3.0mg/mL oxLDL, the highest concentration used. Most cells at this concentration, in both cell types, appear to have passed through apoptosis and begun secondary necrosis. Figure 84 shows THP-1 cells. Most cells here are swollen, some to a very large size. Plenty of debris is also visible. There are a few smaller, brighter cells remaining. The U937 figures are similar. Figure 85 was photographed at only 40x magnification, so a greater area can be seen. Many cells have clumped together and spilled their contents, and a few bright cells, separate from the others, are still present. Figure 86 shows a close-up of a necrotic cell releasing its contents. Many of the other cells look diffuse.

Morphologically, both U937 and THP-1 cells appear to be undergoing apoptosis with oxLDL. Higher concentrations cause some cells to become necrotic, or to pass through apoptosis to secondary necrosis. 78NP appears to be able to inhibit the development of these characteristics of apoptosis in U937 cells, but not THP-1 cells.

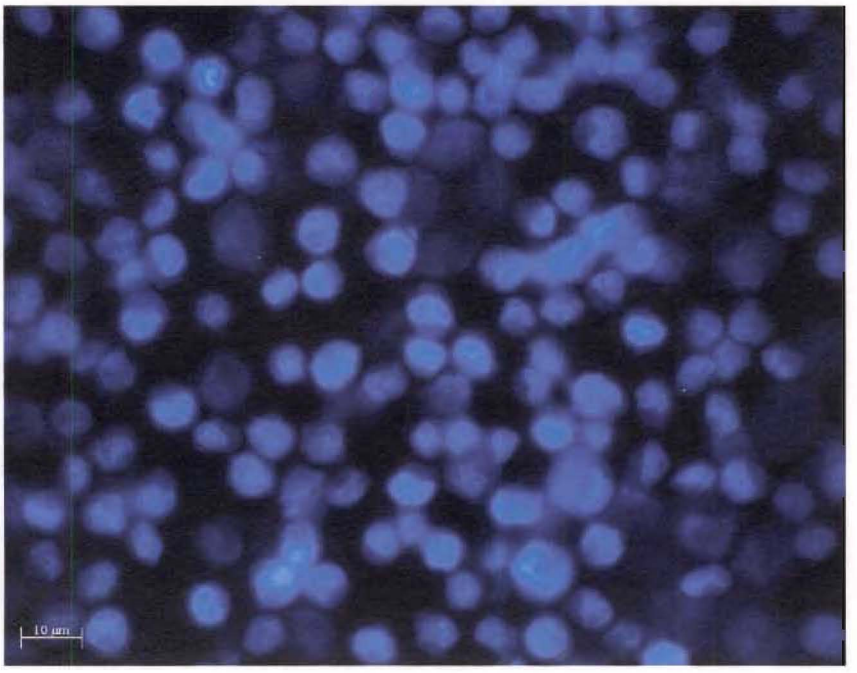

Figure 74: THP-1 cells (5 x 10^5 cells/mL) were incubated in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken on a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope.

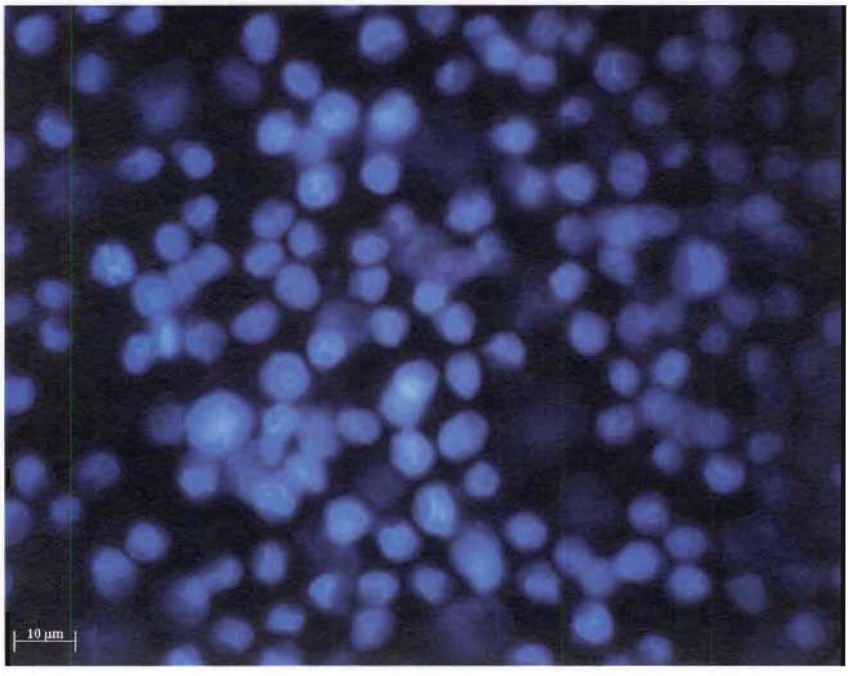

Figure 75: U937 cells (5 x 10^5 cells/mL) were incubated in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken on a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope.

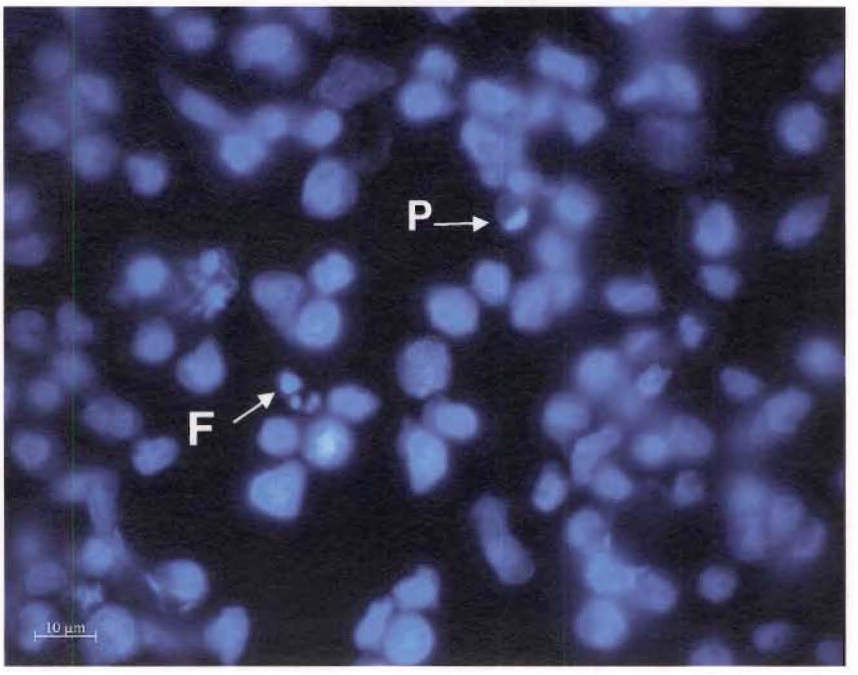

Figure 76: THP-1 cells (5 x 10^5 cells/mL) were incubated with 0.5mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. P indicates an example of pyknosis and F fragmentation.

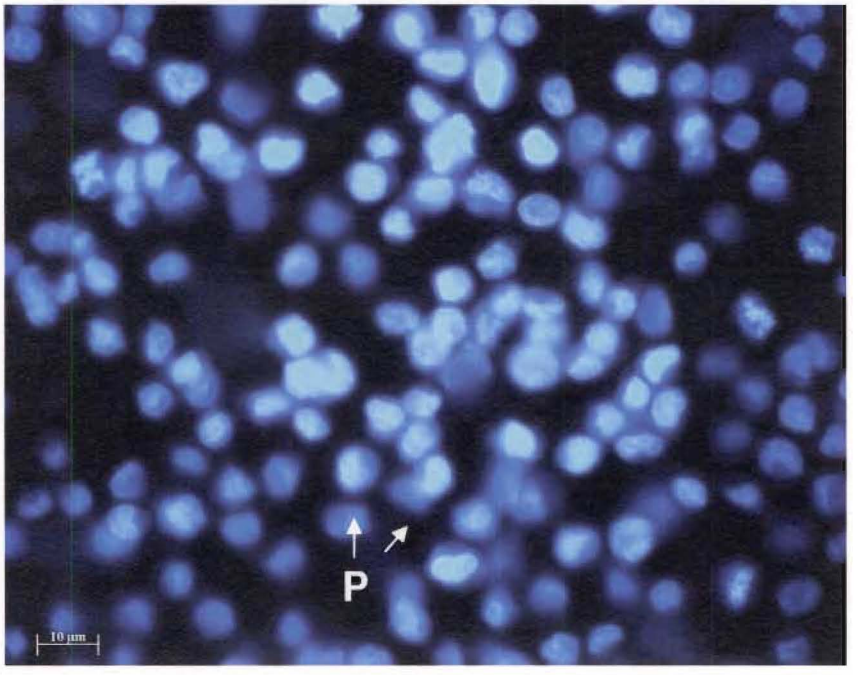

Figure 77: U937 cells (5 x 10^5 cells/mL) were incubated with 0.5mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. P indicates examples of pyknosis.

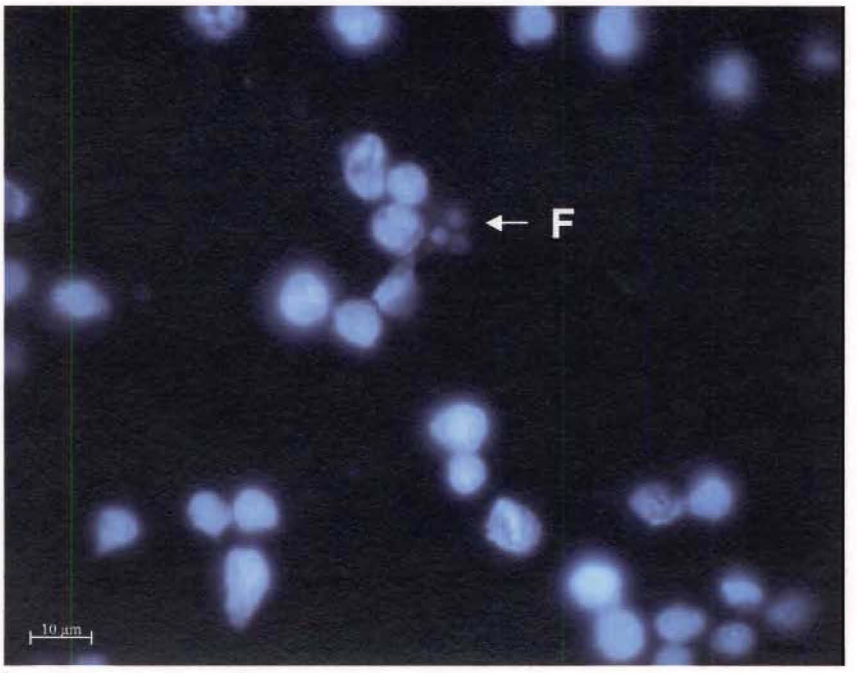

Figure 78: THP-1 cells (5 x 10^5 cells/mL) were incubated with 1.5mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. F shows an example of fragmentation.

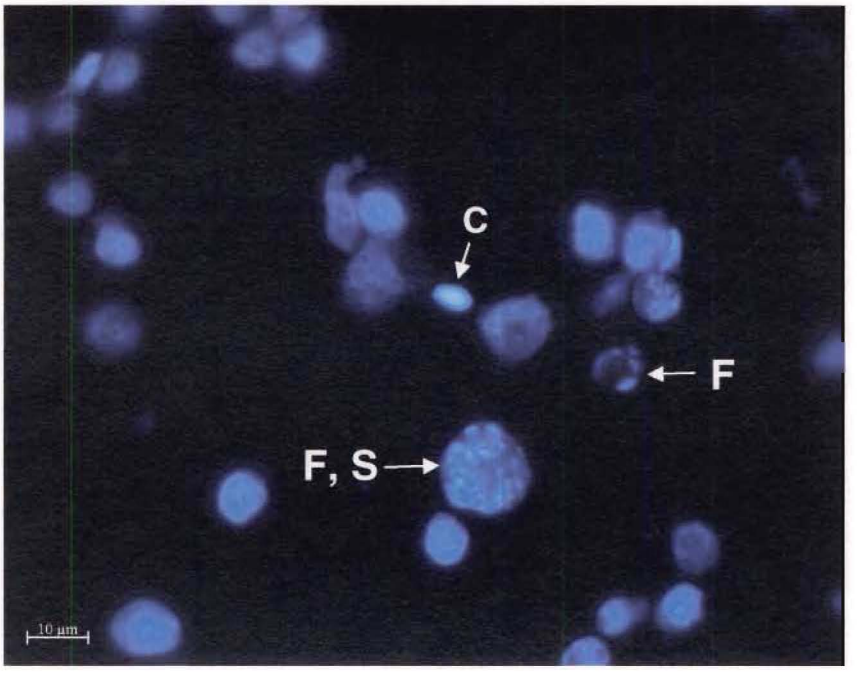

Figure 79: THP-1 cells (5 x 10^5 cells/mL) were incubated with 1.5mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. F indicates examples of fragmentation, C represents condensation and S shows an example of swelling.

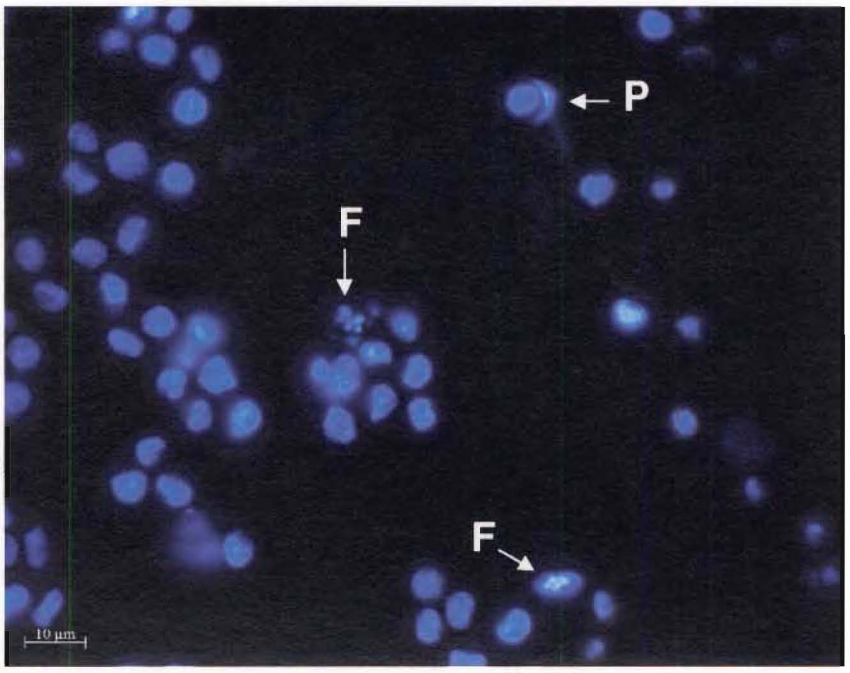

Figure 80: U937 cells (5 x 10^5 cells/mL) were incubated with 1.5mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. F indicates examples of fragmentation and P shows pyknosis.

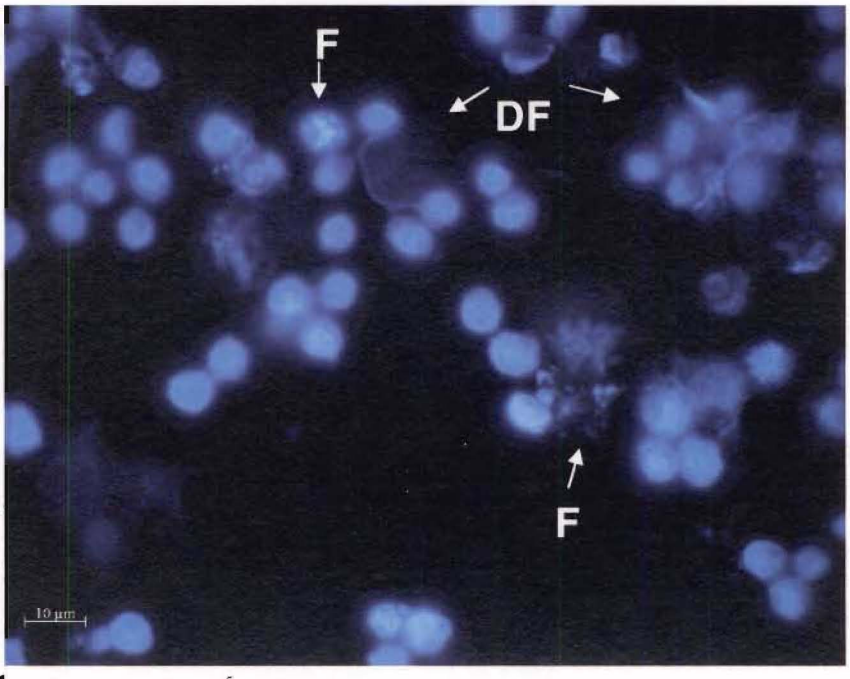

Figure 81: U937 cells (5 x 10^5 cells/mL) were incubated with 1.5mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. F indicates examples of fragmentation and DF shows diffuse cells.

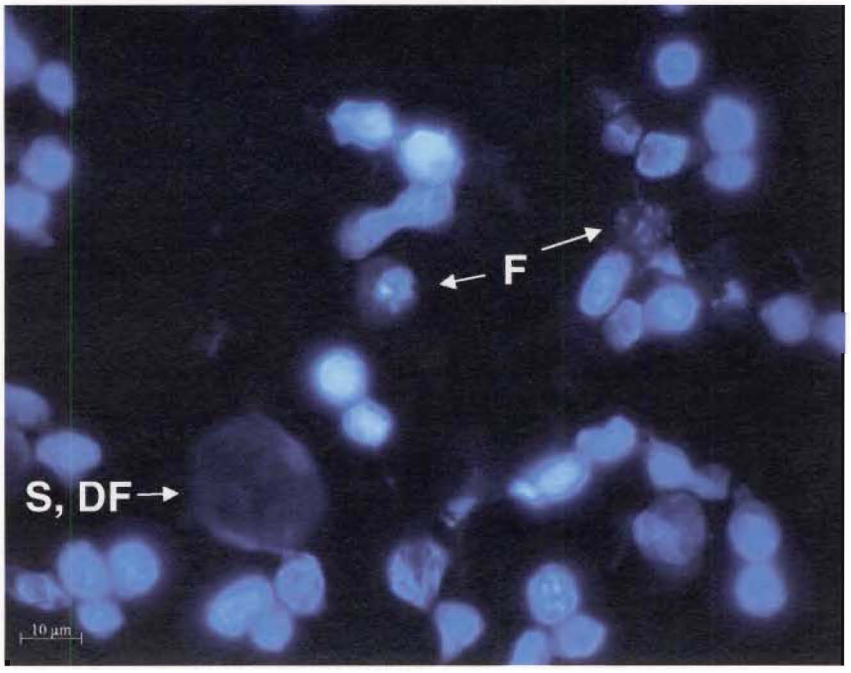

Figure 82: THP-1 cells (5 x 10^5 cells/mL) were incubated with 1.5mg/mL oxLDL and 200µM 78NP in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. F indicates examples of fragmentation and S and DF show a swelling, diffuse cell.

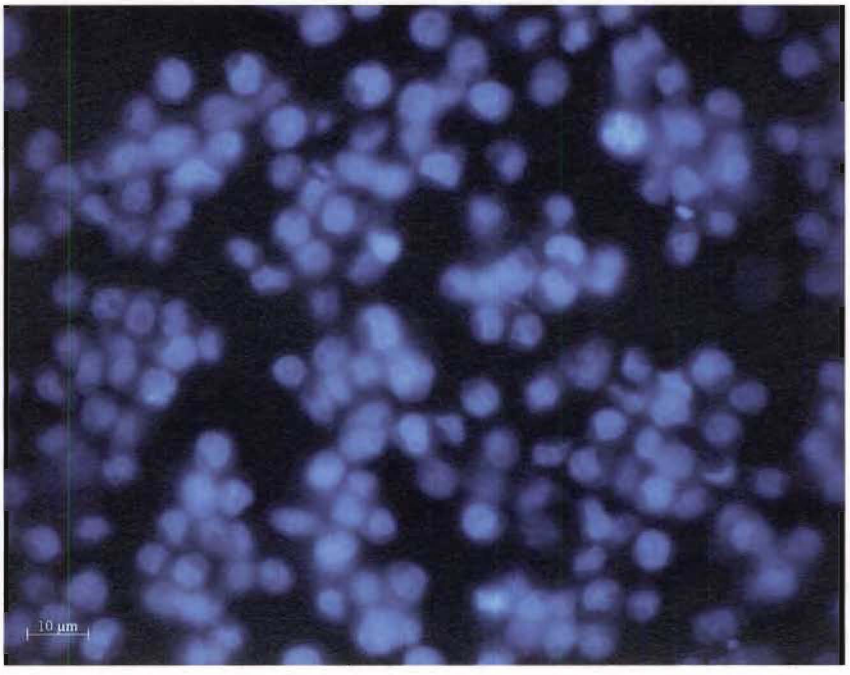

Figure 83: U937 cells (5 x 10^5 cells/mL) were incubated with 1.5mg/mL oxLDL and 200µM 78NP in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope.

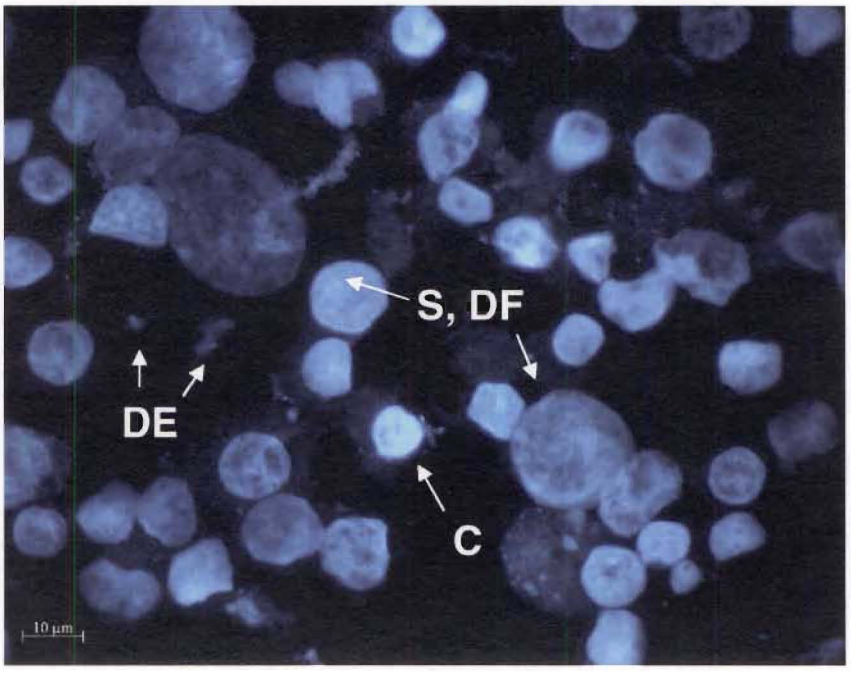

Figure 84: THP-1 cells (5 x 10^5 cells/mL) were incubated with 3.0mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. C indicates an example of condensation. S and DF show swelling and diffuse cells and DE indicates cell debris.

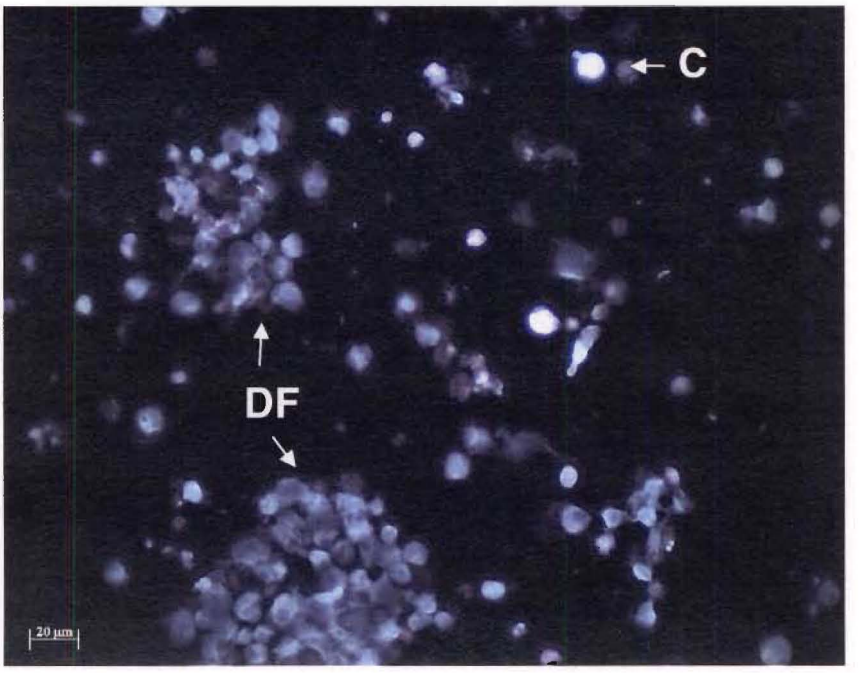

Figure 85: U937 cells (5 x 10^5 cells/mL) were incubated with 3.0mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken at 40x magnification using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. C indicates an example of condensation and DF shows clumps of diffuse cells.

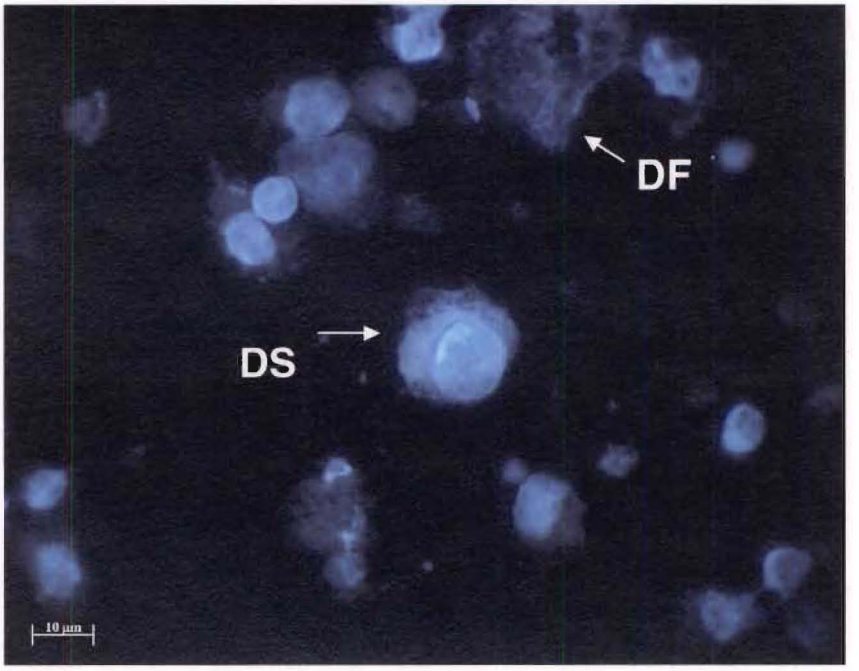

Figure 86: U937 cells (5 x 10^5 cells/mL) were incubated with 3.0mg/mL oxLDL in RPMI 1640 for 24 hours. The cells were washed in PBS, fixed and stained with Hoechst 33342. The picture was taken at 100x magnification using a Zeiss Axiocam camera attached to a Zeiss fluorescence microscope. DS indicates an example of a disintegrating cell and DF shows a diffuse cell.

THE DEFINITION OF APOPTOSIS

According to the Hoechst and Annexin V/propidium iodide assays, both U937 and THP-1 cells are undergoing apoptosis with oxLDL, at least at the two lower concentrations tested. The cells then appear to undergo secondary necrosis, which is a possibility given the lack of phagocytes to clear the apoptotic monocytes. Some primary necrosis may also be present. However, caspase activation, often seen as the cornerstone to a description of apoptosis, is not as conclusive. THP-1 cells show caspase activation exactly as expected. U937 cells, though, have very low levels of caspases, which decrease even further as time of incubation or oxLDL concentration increases.

An issue that is important to consider at this point is which events are required before a cell can be said to be undergoing apoptosis, as opposed to necrosis. There are three main differences between the apoptotic and necrotic death processes, which may help in pinning down their definitions.

To begin with, apoptosis is a biochemically active metabolic process. The fact that some of the apoptotic triggers include changes in concentrations of growth factors and cytokines are indications of this (Schobersberger *et al.*, 1996). It requires ATP for chromatin condensation and DNA fragmentation, and caspase activation by APAF-1 (Lelli Jr. *et al.*, 1998; Carmody and Cotter, 2001). The energy-requiring period of apoptosis after exposure to staurosporin or anti-Fas antibody is 90 minutes (Leist and Nicotera, 1997).

In contrast, necrosis is a passive process, which does not require ATP (Leist and Nicotera, 1997). The artificial lowering of ATP results in necrosis, even under conditions where apoptosis would usually predominate, such as in the cases of T cells with etoposide or anti-Fas antibody, or endothelial cells with hydrogen peroxide (Lelli Jr. *et al.*, 1998). In liver damage by TNFα or Fas, apoptosis occurs early on, but after extended exposure, when ATP has been depleted, necrosis is the dominant form (Leist and Nicotera, 1997).

Secondly, apoptosis is a highly regulated process, initiated intentionally by the cell when it considers itself beyond repair. The high degree of regulation is illustrated in human endothelial cells upon addition of HOCl. Half of the HOCl is gone in the first minute, but the apoptosis not seen until hours later (Vissers *et al.*, 1999).

Necrosis is therefore used as a default method of cell death, when apoptotic regulation fails, such as when apoptotic signals are blocked or anti-apoptotic proteins over-expressed, as well as in cases where cell injury is sudden and severe or too much energy is lost (Leist and Nicotera, 1997; Alcouffe *et al.*, 1999). Since necrosis is not a well-regulated process, once embarked upon, it cannot be converted back to apoptosis (Palomba *et al.*, 1996).

The other major difference between apoptosis and necrosis is the implication of death on neighbouring cells (Leist and Nicotera, 1997). Apoptotic bodies are tidily phagocytosed, whereas necrotic cells lyse, releasing debris which can cause an inflammatory response (Lelli Jr. *et al.*, 1998).

The parts of the apoptotic process responsible for these differences will be important in attempting to define apoptosis. DNA fragmentation, which requires ATP, the activation of caspase enzymes as part of the regulation and execution, and the externalization of phosphatidylserine to induce phagocytosis are three features that could therefore be considered as critical markers of apoptosis. These were all explored in this work.

The problem with using DNA fragmentation and caspase activity as definitions for apoptosis is that they are not always present in every apoptotic-like situation.

Endonuclease activity and their alterations to the nucleus appear not to be essential for apoptosis, since inhibitors of endonucleases such as aurintricarboxylic acid, Zn^{2+} ions and Ca^{2+} chelators reduce cytotoxicity and retard death, but do not prevent it (Reid *et al.*, 1993B; Zamzami *et al.*, 1995; Escargueil-Blanc *et al.*, 1997). In fact, full DNA fragmentation does not feature in all forms of apoptosis, and sometimes only single strand nicking is found. This is usually the case in carcinoma cell lines and hepatocytes (Ueda and Shah, 1994).

Splenocytes, U937 cells and WEHI-231 pre-B cells were found to be irreversibly committed to apoptosis, without nuclear damage (Zamzami *et al.*, 1995). J774 cells with oxLDL showed morphological changes associated with apoptosis, but had no DNA fragmentation (Hakamata *et al.*, 1998).

A persistent dogma of apoptosis is that its execution absolutely requires caspases. There are many proteins able to act as triggers of apoptosis, but the final trigger, that signals the point of no return, has often been described as caspase-3. Caspase inhibitors have usually been reported to be successful at completely stopping apoptosis, as they would be expected to be if caspases are the only final trigger point. Other cells, incubated with apoptotic stimuli

and caspase inhibitors such as ZVAD.fmk or p35, will still undergo apoptosis, but in a form without caspase cleavage, chromatin condensation or DNA laddering. Some of these inducers are puromycin, NO, irradiation and the overexpression of Bax (Borner and Monney, 1999).

In cells from caspase-3 knockout mice, the requirement for caspase-3 was found to be tissue and stimulus specific. It was dispensable in immature T and B cells, but differentiated T cells were resistant to UV-irradiation without it, although still susceptible to γ-irradiation (Woo *et al.*, 1998). In human hepatoma $HepG_2$ cells incubated with *Salvia miltiorrhiza*, there was no procaspase-3 cleavage, and a general caspase inhibitor decreased apoptosis by only 40% (Liu *et al.*, 2001). Neutrophils undergoing an apoptotic-like process with PMA had no DNA fragmentation or caspases (Fadeel *et al.*, 1998; Hampton *et al.*, 2002C). Caspase inhibitors could not suppress apoptosis in myeloid cells incubated with doxorubicin or vincristine (Lotem and Sachs, 1996).

It is possible that some caspases not yet found may be resistant to the inhibitors, or the inhibitors may be unstable and degraded within cells (Borner and Monney, 1999).

It is more likely that another system may work in place of the caspases, such as the events associated with the mitochondria or lysosome. This might involve AIF or serine proteases, calpains or cathespins, which also degrade cellular proteins, but in a less efficient manner. In MRC-5 cells and human lung cells incubated with cadmium, apoptotic characteristics including mitochondrial depolarization, chromatin condensation, DNA fragmentation and phosphatidylserine exposure were found. There was no caspase activity, but AIF was found to be translocated to the nucleus (Shih *et al.*, 2003). Serine proteases have been found to have a role in early chromosomal digestion. Inhibition of serine protease AP24 conferred better protection against UV apoptosis than a caspase-3 inhibitor (Borner and Monney, 1999).

Calpains have been found to be part of apoptosis due to hypoxia in hepatocytes and TNFα-induced apoptosis in U937 monocytes, which featured phosphatidylserine exposure, plasma membrane blebbing and nuclear fragmentation. It was inhibited by calpain inhibitor calpeptin (Vanags *et al.*, 1996). Calpains may be activated by a Ca^{2+} rise. They are able to cleave fodrin and actin-binding proteins such as α-actinin, eliminating anchorage to the membrane and leading to blebbing. They also degrade protein kinases and transcription factors, as well as each other (Borner and Monney, 1999; Kinoshita *et al.*, 1999).

OxLDL-induced leakage of the lysosome would provide DNases and cathespin enzymes, which might act as executioners (Brunk *et al.*, 1997; Ishisaka *et al.*, 2001). Cathespin D is involved in apoptosis induced by IFNγ, Fas and TNFα and provokes apoptosis when overexpressed (Borner and Monney, 1999). Inhibitors of cathespins B and L could suppress apoptosis in myeloid cells incubated with p53 or cycloheximide or undergoing γ-irradiation, and adding caspase inhibitors did not have an additional protective effect (Lotem and Sachs, 1996). The range of cathespin enzymes may mean they are able to be effective under many different conditions. Cathespin B is a thiol protease, which might therefore be inactivated by reduced thiol loss, but cathespin D, an aspartic acid protease, might still be active (O'Neil *et al.*, 1997).

OxLDL can induce cathespin D (but not B or L) in human macrophage-derived foam cells and in lipid-rich atherosclerotic lesions, and the activity of several of the lysosomal hydrolases has been found to increase two-to-four-fold compared to normal arteries or fibrosed or complicated lesions (Wei *et al.*, 1998).

In sphingosine-mediated apoptosis in Jurkat and J774 cells, lysosomal protease activity preceded caspase activation and changes of mitochondrial membrane potential. Lysosomal protease inhibitors inhibited caspase activity (Kagedal *et al.*, 2001). Cathespin B activity also preceded apoptotic features in WEHI-S fibrosarcoma cells incubated with TNFα. Cathespin B inhibitors, but not caspase inhibitors, decreased apoptosis (Foghsgaard *et al.*, 2001).

The other feature examined in this chapter as part of apoptosis is the externalisation of phosphatidylserine. This is particularly important in considering apoptosis in an *in vivo* context, rather than just in the cell as an individual unit. Phosphatidylserine exposure influences how the cell interacts with and affects other cells and components which surround it in the body. It can be argued that this is the most important component of apoptosis, since cells can be cleared without releasing their inflammatory contents (Leist and Nicotera, 1997; Hampton *et al.*, 2002C). If phagocytosis does not occur, the cell will undergo necrosis, and the careful regulation of apoptosis will have lost what is perhaps its major function. Necrotic cells may also be taken up by phagocytes, but they still cause pro-inflammatory responses (Hirt *et al.*, 2000; Cocco and Ucker, 2001). No examples have been located of apoptosis in

which phosphatidylserine exposure is not present. However, it is not a feature as often studied as DNA fragmentation or caspase activation.

The boundaries between apoptosis and necrosis are also blurred due to the fact that the type of cell death is so very dependent on the exact circumstances. The same stimulus can induce apoptosis or necrosis or both, even simultaneously in the same cell population. This will vary depending on stimulus, the concentration of the stimulus, cell type and length of incubation. Necrotic features may simply represent the end stages of apoptosis *in vitro*, as phagocytosis cannot usually occur. No consistency is found between cell types, or even within the same cell type with different inducers.

The two processes also have many common features, which makes them less distinguishable from one another. Among these are the involvement of Ca^{2+}-dependent enzymes, c-Fos and mitochondrial membrane permeability (Kroemer and Reed, 2000). There is limited evidence of caspases-8 and -10 participating in necrosis, and that Bcl-2 overexpression inhibits necrosis by viruses, oxidative stress and hypoxia (Leist and Nicotera, 1997; Hetz *et al.*, 2002), in spite of these being considered strict indicators of apoptosis. This has not been confirmed independently and is ignored by most authors.

Annexin V is able to gain access to phosphatidylserine during necrosis as a result of membrane fragmentation. The Annexin V/PI assay is based upon distinguishing the two forms of cell death by loss of cell membrane integrity (Vermes *et al.*, 1995; Green and Steinmetz, 2002). However, it has been suggested that phosphatidylserine may be exposed on necrotic cells for a short time before the membrane integrity is lost (Waring *et al.*, 1999; Lecoeur *et al.*, 2001).

A new view is emerging that, instead of completely separate phenomena, apoptosis and necrosis are only extremes in a wide range of biochemical and morphological patterns of cell death (Lelli Jr. *et al.*, 1998; Borner and Monney, 1999). Apoptosis has previously been used as a heterogeneous umbrella term, to pull together a set of events which often occur together, and necrosis was usually defined as the absence of these. The idea of a continuum between two polar forms of cell death, as opposed to a dichotomy, would allow for examples missing so-called 'crucial' apoptotic features such as caspases or DNA fragmentation. These

forms would therefore be placed somewhere towards the middle of the spectrum, depending on what other characteristics the cell death demonstrated.

The three characteristics tested for in this chapter are all important parts of the death process. Together, they would make a strong case for apoptosis, as they look at different parts of the process, across the timeframe and different functions that are carried out as part of it. From this, it can be concluded that THP-1 cells are undergoing a conventional form of apoptosis with oxLDL, and that U937 cells are also likely to become apoptotic, but their death with oxLDL is more towards the middle of the scale.

Perhaps a range of assays like this is required before apoptosis can be diagnosed. So many features have been identified as part of apoptosis, most, but not all, of which were outlined in the introductory chapter. Many of these are not tested for by those undertaking apoptotic studies. As a result, many of the literature examples of apoptosis may in fact be towards the middle, rather than one end, of the spectrum, as they may be missing many important apoptotic characteristics.

SUMMARY

Three assays were used to assess the appearance of apoptosis in THP-1 and U937 cells with oxLDL. The Hoechst 33342 stain was used to examine changes in nuclear morphology, and it was found that both cell types showed the same typical apoptotic features. Higher concentrations of oxLDL caused either necrosis or secondary necrosis as well.

The Annexin V/propidium iodide assay looked at phosphatidylserine exposure on the surface of the cells. Both cell types seemed to undergo apoptosis, with THP-1 cells moving more swiftly towards (secondary) necrosis.

A fluorescent caspase assay was used to test for caspase activation. Here the two cell types were very different. THP-1 cells had an increase in caspase activity over time and with oxLDL concentration, although this then dropped away as secondary necrosis began to take place. The 3.0mg/mL oxLDL concentration was found to cause more necrosis than apoptosis.

In U937 cells, the caspase levels were very low, and further incubation with oxLDL lowered them still further. A correlation of loss of total reduced thiols and loss of caspase activity led to an demonstration of oxLDL inactivation of already activated caspases, suggesting this may be one of its effects in U937 cells.

78NP seemed to reduce the appearance of apoptotic features in U937 cells as measured by Hoechst 33342 staining and the Annexin V/propidium iodide assay, and continued to have no effect on THP-1 cells. It was able to partly decrease the loss of caspase activity occurring in U937 cells when the oxLDL was added, bringing them up closer to control levels. This may be related to its protection of reduced cellular thiols, shown by the DTNB assay in earlier chapters.

78NP PRODUCTION BY IFNγ *IN VIVO* AND CELL VIABILITY

INTRODUCTION

IFNγ, generated by T cells in inflammatory conditions such as in the atherosclerotic plaque (Wachter *et al.*, 1989), has important roles throughout atherosclerotic development. Some of these effects may be due to the production of 78NP.

Stimulation of cells with IFNγ reduces their ability to oxidise LDL. In mouse macrophages, this is via increased NO synthesis, and lipopolysaccharide, which also stimulates iNOS, can have the same effect (Bolton *et al.*, 1994). In human mononuclear cells, reduction of LDL oxidation by IFNγ may be due to contributions from 78NP, 3-hydroxyanthranilic acid (3HAA), a tryptophan metabolite found in mammalian cells (Werner-Felmayer *et al.*, 1989; Christen *et al.*, 1990; Thomas and Stocker, 1999) or unidentified metal ion chelators (Baoutina *et al.*, 2001A). 3HAA at 10μM has been reported to decrease cell-mediated LDL oxidation when added to cultures. Inhibition of the flavin enzyme responsible for 3HAA and lower levels of tryptophan in the medium than required for 3HAA formation halted the inhibition by IFNγ (Christen *et al.*, 1994). Addition of 5μM 78NP to media inhibited TBARS formation in LDL caused by THP-1 monocytes, whereas 20μM was required with THP-1 macrophages to stop lipid oxidation and reduce α-tocopherol loss (Gieseg and Cato, 2003). IFNγ can also inhibit lipoxygenase activity by around 20%, which might reduce its contribution to LDL oxidation (Fong *et al.*, 1994; Folcik *et al.*, 1997).

IFNγ can decrease foam cell formation. Stimulation of human monocyte-derived macrophages with IFNγ for 3 days downregulated scavenger receptor A at the mRNA level, modestly reducing the number of receptors on the surface of the cell, with a concomitant reduction in acLDL binding and internalization, decreased cholesterol accumulation and inhibition of the transformation into foam cells (Fong *et al.*, 1990; Geng and Hansson, 1992). IFNγ may affect the scavenger receptor by inducing Stat1 binding to the promoter, antagonising the AP-1 and *ets* transcription factors, and possibly also destabilising the mRNA (Grewal *et al.*, 2001). IFNγ also regulates other oxLDL receptors, such as CD36 (Nicholson *et al.*, 1995A). IFNγ affects the metabolism of the modified LDL as well, slowing the

movement of acLDL to the lysosomes in peritoneal mouse macrophages, by controlling microtubules and their regulation of all intracellular movement (Fong *et al.*, 1990).

IFNγ may conversely promote foam cell formation. It has been shown to reduce cholesterol efflux by downregulating the ATP-binding-cassette-1 transporter gene, which is used for apoA-I and apoE mediation of efflux. In human monocyte-derived macrophages it decreases apoE production (Garner *et al.*, 1997A; Panousis and Zuckerman, 2000A). It is associated with increased intracellular cholesteryl ester accumulation and induces ACAT expression and activity in peritoneal murine macrophages. It also increases lipoprotein receptors on smooth muscle cells, enhancing their foam cell formation (Panousis and Zuckerman, 2000B).

IFNγ might also delay apoptosis, reducing foam cell death. Three studies showed IFNγ reducing apoptosis in M1 myeloid leukemic cells transfected with p53, or incubated with cycloheximide and doxorubicin. Other cytokines such as IL-6, IL-3 and GM-CSF could have the same effect. Intracellular oxidative stress levels were not decreased, but caspase activation was. IFNγ could also act in synergy with caspase inhibitors, diminishing apoptosis levels still further (Lotem *et al.*, 1996; Lotem and Sachs, 1996; Lotem and Sachs, 1998).

If 78NP acts to protect the monocytes and macrophages in inflammatory situations such as the atherosclerotic plaque, the cells must produce 78NP at a high enough concentration for it to act as an antioxidant. The production of 78NP by the three cell lines used in this work during incubations with IFNγ is examined.

Previous studies (Table 4) have investigated the production of neopterin by IFNγ in monocytes or macrophages. Troppmair (1988) uses a radioimmunoassay for neopterin, whereas all others use the HPLC pterin method. Troppmair's experiments examined only the supernatant (amount of neopterin excreted by the cell) and gave results as medians. Most of the rest of the studies looked at the neopterin contained within the cell, which was analysed after cell lysis and protein determination.

The cell types all had similar levels of neopterin. All cells produced some neopterin without stimulation from IFNγ, and this level increased markedly once the IFNγ was added. Higher levels of IFNγ resulted in more neopterin production. Monocytes (both peripheral

blood mononuclear cells and THP-1 cells) were found to produce more neopterin before stimulation than the macrophages.

After incubation with IFNγ, comparisons between cell types become less clear. From Troppmair's measurements, it appears that monocytes produce less neopterin after addition of IFNγ than macrophages. A similar assessment of the remaining studies is complicated by the dissimilarities in THP-1 monocyte measurements by Weiss (1992) and Werner-Felmayer (1990). Weiss' value for neopterin after incubation of cells with 300U/mL IFNγ is around fourfold more than Werner-Felmayer's result with 250U/mL IFNγ. Therefore macrophages may produce more or less neopterin after incubation with IFNγ than monocytes, depending on whether the values from Weiss or Werner-Felmayer are considered.

The discrepancy is intriguing, since these results came from closely collaborating researchers. This suggests that variation in neopterin levels produced is common in and between these experiments.

A pattern has been found over time in neopterin generation induced by IFNγ. The production increases and peaks. Troppmair (1988) found this occurred with peripheral blood macrophages by 4 days, and *in vivo* after IFNγ injection by 48 hours. The amount of neopterin would then level out, or decrease.

Werner-Felmayer (1990) showed that THP-1 monocytic cells have the same pteridine production patterns as peripheral blood macrophages, producing similar amounts of neopterin, monapterin and biopterin. This should make them a suitable model for studying neopterin production *in vivo*.

Table 4: Amounts of neopterin produced by monocytes and macrophages with and without IFNγ in previously published studies.

	THP-1 monocytes (Weiss *et al.*, 1992)	
	Supernatant (nM, 10^6 cells)	Inside cell (pmol/mg cell protein)
Without IFNγ	3.8±0.6	12.6±3.1
300U/mL IFNγ	81.7±4.9	206.0±22.3

	THP-1 monocytes (Werner-Felmayer *et al.*, 1990)
	Inside cell (pmol/mg cell protein)
Without IFNγ	4.7±4.0
250U/mL IFNγ	52.1±0.8
500U/mL IFNγ	150.5±9.2
1000U/mL	334.9±14.8

	Peripheral blood mononuclear cells (Troppmair *et al.*, 1988)	Monocyte-derived macrophages (Troppmair *et al.*, 1988)
	Supernatant (pmol/10^6 cells)	Supernatant (pmol/10^6 cells)
Without IFNγ	22	14
100U/mL IFNγ	79	189

	Monocyte-derived macrophages (Werner *et al.*, 1989)	Monocyte-derived macrophages (Werner-Felmayer *et al.*, 1990)
	Inside cell (pmol/mg)	Inside cell (pmol/mg)
Without IFNγ	1.95±1.27	2.8±1.9
250U/mL IFNγ	159.63±51.22	131.4±68.0

In the second part of this chapter, IFNγ is added to cells with oxLDL, to test whether the 78NP produced might protect the cell from viability loss. However, this may also alter viability in other ways, since IFNγ is likely to have numerous other effects aside from 78NP production.

In many cases interferons cause differentiation of the cell as part of the activation process. For instance, an increase in adherence of THP-1 monocyte cells was found after 24 hours of treatment with IFNγ, and *HLA-DR* and *FcRI* expression were enhanced. This was only a partial effect, however, since the cells did not completely differentiate or enlarge to

macrophage size (Tominaga *et al.*, 1998). IFNγ at 100U/mL was said to differentiate U937 cells after 48 hours (Nonaka *et al.*, 1999). When incubating cells with IFNγ in this research, no extra adherence or change in shape which might indicate differentiation was observed, although no specific tests for differentiation markers were undertaken.

IFNγ may have slightly different effects on the THP-1 and U937 cell types. IFNγ induces the *HLA-DR* gene very strongly in THP-1 cells, and weakly in U937 cells, where it is not expressed constitutively. IFNγ also induces TNFα , but only in THP-1 cells (Arenzana-Seisdedos *et al.*, 1988). THP-1 cells' release of ceruloplasmin is said to be somewhat stimulated by IFNγ, whereas U937 cells, which may not constitutively release it, are stimulated twenty-fold by IFNγ (Mazumder *et al.*, 1997). IFNγ also decreases CD4 antigen levels on cells. In U937 cells the reduction was 50%, but the effect was much smaller in THP-1 cells (Faltynek *et al.*, 1988).

In the last part of the chapter, 78NP levels *in vivo* are examined. During inflammation, the metabolites of macrophages and neutrophils damage cells. The resulting accumulation of dead cells, cellular debris and fluid at the site is known as pus (Davies, 1997). Serum neopterin and 78NP is hypothesised to come from such inflammatory lesions, which suggests pterins have been diluted from sites of much higher concentrations (Duggan *et al.*, 2002).

Levels of 78NP and neopterin have not been measured in bodily materials other than serum to date. Neopterin has been identified in plaque samples by immunoblotting (Reibnegger, personal communication, 2003). Concentrations in serum vary, but stay within the low nanomolar range in healthy subjects (Wachter *et al.*, 1992), in a ratio of neopterin: 78NP of 1:2 (Weiss *et al.*, 1993B). One study with 662 subjects reported serum levels in healthy subjects which varied by age. Those aged 0-18 had 6.78±0.22nM, those aged 19-75, 5.34±0.14nM, and those aged older than 75, 0.67±0.79nM (Wachter *et al.*, 1989). Other studies showed a normal range of 6.2-10nM (Gurfinkel *et al.*, 1999), 3.8-12.2nM (Schumacher *et al.*, 1992) or 5.3±2.7nM (Murr *et al.*, 1999). Some studies reported median concentrations of 4.7nM neopterin and 8.9nM 78NP (Weiss *et al.*, 1994) and 4.85nM neopterin and 6.9nM 78NP (Widner *et al.*, 1999). An upper limit for healthy subjects in another study was given as 15nM (Mueller *et al.*, 1991).

In severely ill patients, serum levels of up to 1 μM have been reported (Baier-Bitterlich *et al.*, 1996B). This is close to the range required for antioxidant activity, as found for prevention of LDL oxidation (Gieseg and Cato, 2003).

Serum levels of neopterin have been examined in atherosclerotic patients. Not all patients have elevated neopterin levels, and the increase in concentration compared to controls is not large, which does not support the contention that 78NP might play an important role in plaque development. The lower elevation of neopterin in some patients may reflect varying levels of inflammation, and so 78NP production, in the plaque.

In one study, the average neopterin level was 15.5±19.35nM, close to the upper limit of the controls. Half of the patients had elevated levels, with 20% above 20nM and one up to 113.5nM (Tatzber *et al.*, 1991). Only half of the patients with congestive heart failure examined in another study had elevated neopterin levels, and the increase was small (Fuchs *et al.*, 1993). In another group of patients with coronary artery disease or peripheral artery disease, elevations were again very small, with only a few up to around 20nM (Erren *et al.*, 1999). Only 39% of patients with unstable angina had elevated neopterin levels (Gurfinkel *et al.*, 1999). Neopterin was said not to be a specific marker in a group of males with coronary artery disease diagnosed angiographically. The average neopterin levels for patients and controls were 8.0±2.7nM and 7.6±2.5nM respectively (Schumacher *et al.*, 1992).

In a larger study with 321 carotid atherosclerosis patients and 240 controls aged 40-79, those with atherosclerosis had higher neopterin levels. In men, patients had 8.5±2.7nM compared to 6.7±2.3nM in controls, and in women the figures were 9.6±3.3nM versus 7.5±2.3nM. The increase in neopterin was found to be significant in all groups except older women. Neopterin was more strongly correlated with atherosclerosis than other risk factors studied, such as blood pressure and fibrinogen levels, but the authors state that since the difference is small, large study groups are required, and neopterin could not be used as a clinical marker for early diagnosis. Its significance was said to be that it adds to the evidence of immune activation in atherosclerosis (Weiss *et al.*, 1994).

RESULTS AND DISCUSSION

A. PRODUCTION OF 78NP BY IFNγ STIMULATION OF MONOCYTES AND MACROPHAGES

The amount of neopterin produced by THP-1 monocytes, U937 monocytes and THP-1 macrophage-like cells after 48 hours incubation with IFNγ is represented in Table 5. The results are presented so as to allow comparison with earlier studies.

Table 5: The amount of neopterin produced by three cell types after stimulation with IFNγ for 48 hours. THP-1 or U937 monocytes (5 x 10^5cells/mL) or THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated in RPMI 1640 with IFNγ (specific activity 2 x 10^7U/mg protein) of 300U/mL or 1500U/mL for 48 hours. The supernatant was then removed, and the cells washed and lysed in water by sonication. The amount of neopterin present was measured using the pterin HPLC assay after oxidation with acidic iodide. Protein determinations were carried out for each cell type. Results are expressed as the mean ± SD of triplicates.

Treatment	U937 monocytes	THP-1 monocytes	THP-1 macrophage-like cells
Supernatant (nM) No IFNγ	20.41±5.50	16.45±1.08	26.82±4.53
Within cells (nmol/mg cell protein) No IFNγ	2.97±0.04	2.36±0.21	4.73±0.71
Supernatant (nM) 300U/mL IFNγ	59.57±10.55	30.75±0.71	53.03±3.71
Supernatant (pmol/10^5 cells) 300U/mL IFNγ	11.91±2.11	6.15±0.14	53.03±3.71
Within cells (nmol/mg cell protein) 300U/mL IFNγ	4.45±0.64	3.64±0.02	11.15±0.93
Supernatant (nM) 1500U/mL IFNγ	90.61±3.95	55.39±4.05	86.19±13.87
Supernatant (pmol/10^5 cells) 1500U/mL IFNγ	18.12±0.79	11.08±0.81	86.19±13.87
Within cells (nmol/mg cell protein) 1500U/mL IFNγ	6.05±0.80	4.56±0.04	18.65±1.11

Under all conditions examined, U937 monocytes produced more 78NP than THP-1 monocytes. This result was unexpected, as the parental U937 cell line was unable to produce

78NP, however certain subclones are known to have undergone changes such that 78NP production does occur (Wachter *et al.*, 1989; Werner-Felmayer *et al.*, 1990). The U937 cell line used in our studies appears to belong to one of these groups.

The THP-1 macrophage-like cells yield by far the largest amount of 78NP, once the number of cells or the amount of cell protein is taken into account. This is not surprising, since one of the effects of differentiation is an increase in IFNγ receptors (Kosaka *et al.*, 2001), and an increase in production of substances associated with inflammation, including antioxidants (Akeson *et al.*, 1991B; Gotoh *et al.*, 1993).

Before the concentrations of neopterin measured were converted from nM into nmol/mg cell protein, it was noted that the concentrations found in the cell lysates, and hence inside the cell, were higher than those in the supernatants.

Another observation is that the five-fold higher concentration of IFNγ did not yield a five-fold higher concentration of 78NP. Instead the increase ranged between around 125% and 180%. The results in Table 4 from a similar experiment (Werner-Felmayer, 1990) show a much more proportional increase. The highest IFNγ concentration tested here is much greater than the one in the table. Either a threshold is reached, over which extra IFNγ has no further effect, or the production levels off, becoming less efficient with increasing concentrations. Such a high concentration may also have decreased 78NP production, or cell viability.

The monocyte cell lines showed overall lower levels of 78NP increase with higher IFNγ concentration, and the smallest increases were found within the cell rather than in the supernatant. The THP-1 macrophage-like cells had approximately even increases both within and outside the cell.

Studies over time, using only 300U/mL IFNγ, showed the pattern of an increase in neopterin concentration which peaked at 24 hours and tailored off (Figures 87 and 88). This trend was also found by Troppmair (1988). The reduction in neopterin concentration may be due to both a decrease in IFNγ-induced production and the oxidation of the 78NP present to products other than neopterin. In Chapter 4, 78NP incubated with cells was shown to oxidise almost exclusively to 7,8-dihydroxanthopterin.

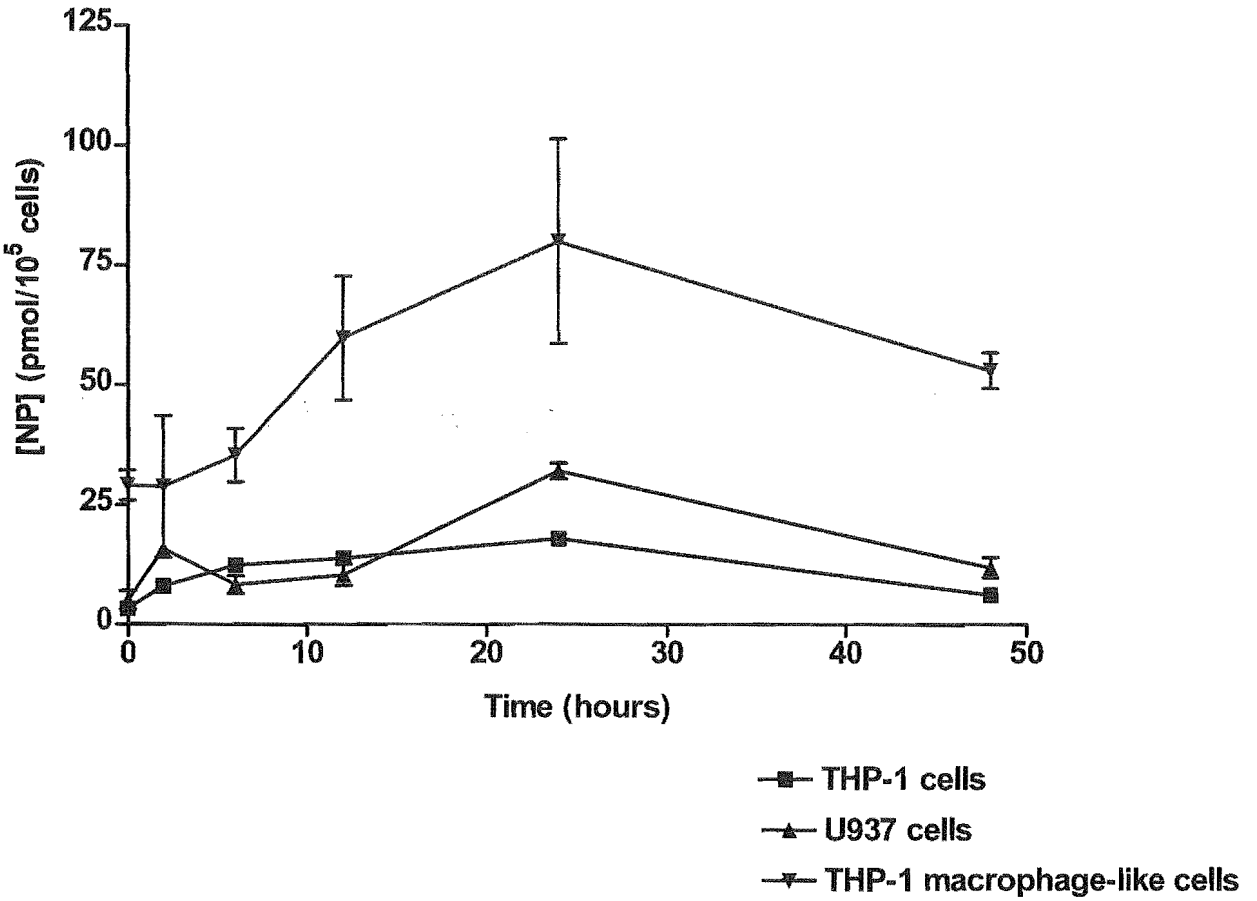

Figure 87: Production of neopterin in IFNγ-stimulated cell supernatant over 48 hours.
THP-1 or U937 monocytes (5 x 10^5cells/mL) or THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated in RPMI 1640 with IFNγ (specific activity 2 x 10^7U/mg protein) of 300U/mL for up to 48 hours. At 0, 2, 6, 12, 24 or 48 hours, the supernatant was removed. The amount of neopterin present was measured using the pterin HPLC assay after oxidation with acidic iodide. This experiment was repeated twice, and the results presented are from a representative experiment. Results are expressed as the mean ± SD of triplicates.

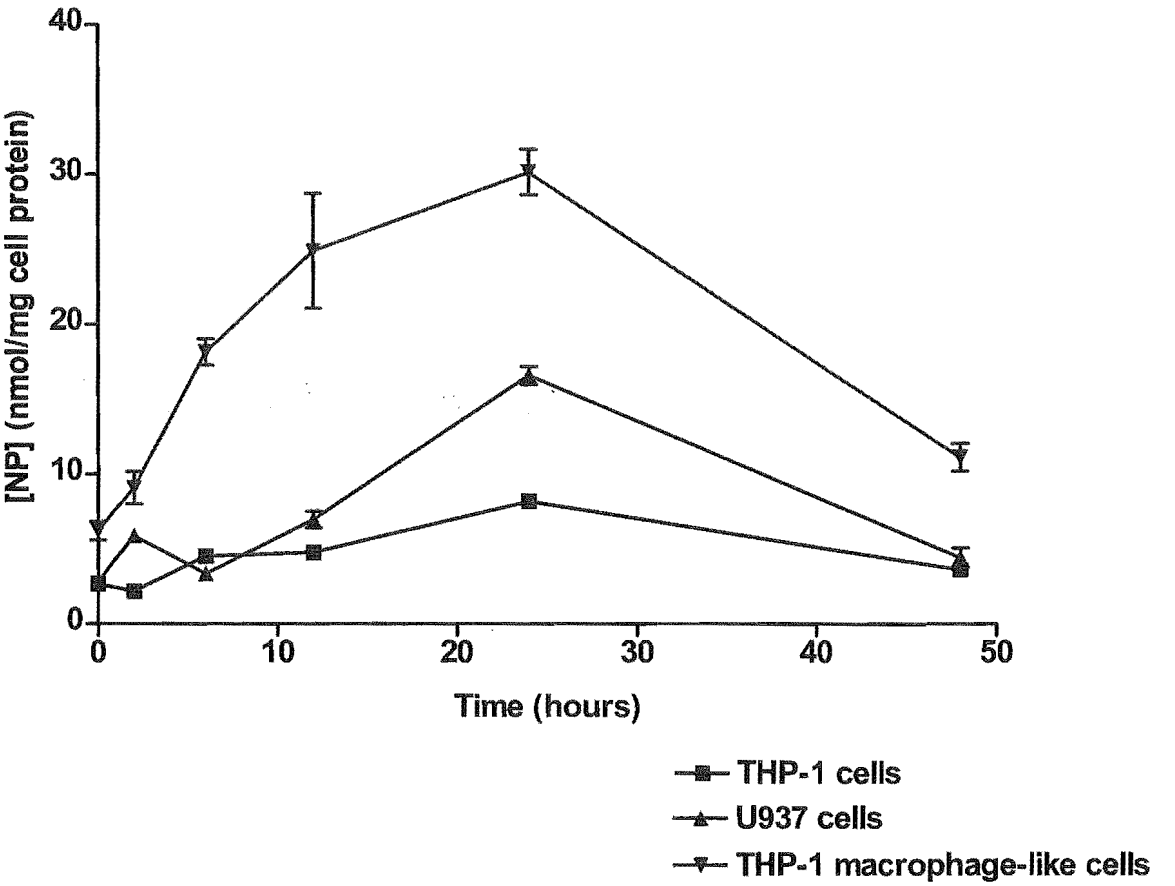

Figure 88: Production of neopterin in IFNγ-stimulated cell lysate over 48 hours.
THP-1 or U937 monocytes (5 x 10^5cells/mL) or THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated in RPMI 1640 with IFNγ (specific activity 2 x 10^7U/mg protein) of 300U/mL for up to 48 hours. At 0, 2, 6, 12, 24 or 48 hours, the supernatant was then removed, and the cells washed and lysed in water by sonication. The amount of neopterin present was measured using the pterin HPLC assay after oxidation with acidic iodide. Protein determinations were carried out for each cell type. This experiment was repeated twice, and the results presented are from a representative experiment. Results are expressed as the mean ± SD of triplicates.

The relative amounts of 78NP produced by each cell type match those in the experiments represented in Table 5. THP-1 macrophage-like cells produced the most 78NP, relative to cell number or amount of cell protein, and U937 cells generated more than the THP-1 monocytic cells. The maximum amount of 78NP produced by each cell type, that is, the amount measured at the 24 hour timepoint, is given in the table below:

Table 6: The amount of neopterin produced by three cell types after stimulation with IFNγ for 24 hours. THP-1 or U937 monocytes (5 x 10^5cells/mL) or THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated in RPMI 1640 with IFNγ (specific activity 2 x 10^7U/mg protein) of 300U/mL for 24 hours. The supernatant was then removed, and the cells washed and lysed in water by sonication. The amount of neopterin present was measured using the pterin HPLC assay after oxidation with acidic iodide. Protein determinations were carried out for each cell type. Results are expressed as the mean ± SD of triplicates.

Treatment	U937 monocytes	THP-1 monocytes	THP-1 macrophage-like cells
Supernatant (nM) No IFNγ	25.50±9.83	16.64±0.68	29.05±3.14
Within cells (nmol/mg cell protein) No IFNγ	2.79±0.36	2.69±0.06	6.23±0.62
Supernatant (nM) 300U/mL IFNγ	160.26±8.29	89.61±1.06	79.94±21.3
Supernatant (pmol/10^5 cells) 300U/mL IFNγ	32.05±1.66	17.92±0.21	79.94±21.3
Within cells (nmol/mg cell protein) 300U/mL IFNγ	16.58±0.60	8.21±0.32	30.17±1.51

The levels of neopterin described in the supernatant were similar, although slightly higher, than those found by Werner (1989), Werner-Felmayer (1990) and Weiss (1992). However the concentrations expressed relative to cell protein, quantifying neopterin inside the cell, were around twenty-fold greater than those in the earlier studies.

A number of reasons could account for the differences. The THP-1 monocytes used by Werner-Felmayer (1990) were passaged only once weekly, as opposed to two or three times for the cells used in these studies. The lower number of cells used here may also have resulted in greater production when cell numbers or protein levels are compared. The earlier studies presented above used THP-1 monocytes at a density of 6 x 10^5/mL, compared to

5 x 10^5/mL in this work. Cell density can affect metabolism (Hughes *et al.*, 1994; Rodriguez *et al.*, 1994).

Since the greatest discrepancy is in the intracellular concentrations, a difference in method used to prepare the cell lysates may be a determining factor. In this study, cells were resuspended in water, after PBS washes. To ensure lysis was complete, the cells were also sonicated for two minutes. A less rigorous protocol, resulting in incomplete cell lysis, would result in smaller 78NP concentrations.

Some 78NP was also produced by unstimulated cells. This level did not increase significantly over time. The THP-1 macrophage cells consistently had a higher level of 78NP present before stimulation with IFNγ, due to their more advanced state of differentiation. This differs from the values given in the studies outlined earlier, in which THP-1 monocytes had higher levels of 78NP before stimulation than monocyte-derived macrophages. The relatively 'undifferentiated' state of the peripheral blood 'macrophages' may have some bearing on this. The cells Werner (1989) extracted from donors were experimented on as 'macrophages' only three days after removal from blood, and so were probably more monocyte-like in nature.

The level of 78NP that was generated is around a thousand-fold lower than that used to protect U937 cells in viability studies in earlier chapters. From the evidence here alone, it seems unlikely that 78NP is able to protect monocytes and macrophages *in vivo*. Of course, *in vivo*, many other antioxidants may be present to contribute to the protection, such as 3HAA, vitamins and antioxidants formed by other cell types. The period of contact with the oxidant will also be much longer, especially in the development of atherosclerosis, which is a slow process, spanning years. Possibly low levels of 78NP, produced continuously over that time due to constant IFNγ stimulation from T cells, are able to aid the cell. Levels of 78NP may also be higher in the complex situation of the plaque due to stimulation of the cells by other cytokines. Another possibility is that when the cell produces the 78NP itself, as opposed to having it exogenously added, it is in a different part of the cell, where it can better act as an antioxidant.

THE EFFECT OF OXLDL ON IFNγ-STIMULATED 78NP PRODUCTION

The effect of oxLDL on IFNγ-induced 78NP production was examined in each of the three cell types many times. Since oxLDL influences so many cell signalling pathways, it is likely to change signalling patterns resulting from IFNγ stimulation. In particular, oxLDL affects the pathway activated by IFNγ once bound to cell-surface receptors, the Janus tyrosine kinases, which phosphorylate Stat factors (Johnson *et al.*, 1994). For example, oxLDL activated Jak2, Stat1 and Stat3 in MRC5 fibroblasts, to a greater degree if it was more extensively oxidised. (Mazière *et al.*, 2001). In THP-1 monocytes, incubation with extensively oxLDL upregulates PPARγ, which then downregulates iNOS and the induction of inflammatory cytokines (Jang *et al.*, 1999). This might be expected to reduce their upregulation by IFNγ (Billiau and Dijkmans, 1990; Tatzber *et al.*, 1991; Kuby, 1997).

The results of adding oxLDL to cells and measuring 78NP production were very variable and irreproducible, although there were trends which appeared most of the time. The results would have been complicated by the other effects the oxLDL was having on the cells at the same time, for instance the decrease in cell viability.

As a general rule, 1.5mg/mL oxLDL added to control cells, not incubated with IFNγ, had no effect on the level of 78NP produced. This was the case even after 48 hours, when a large cell viability loss would have occurred. Sometimes a small increase in the supernatant fraction was found at the 48 hour timepoint, possibly as the 78NP which would have been kept inside the cell was released due to loss of integrity of the cell membrane.

Trends were also apparent in cells incubated with 300U/mL IFNγ when 1.5mg/mL oxLDL was also added. In U937 monocytes, a decrease in 78NP concentration occurred in the supernatant fraction. By 48 hours, an increase was apparent inside the cells. In THP-1 monocytes, most values decreased, both intra- and extracellularly. Occasionally, a small increase was found. In THP-1 macrophage-like cells, any effects seen were very small. There was never a change in the 78NP concentration in the supernatant due to addition of oxLDL. At earlier timepoints, up to 12 hours, a small decrease in 78NP was usually found, whereas in the later stages of the incubation, the concentration increased.

Similar experiments were performed in an examination of THP-1 macrophage-like cells to which non-oxidised LDL was added. LDL alone, at 40nM, caused an increase in

supernatant pterin in IFNγ-stimulated and unstimulated cells, but did not affect intracellular levels (Gieseg and Cato, 2003). In a study of human mononuclear cells, the presence of native or oxLDL did not affect levels of tryptophan degradation or 3HAA synthesis (Christen *et al.*, 1994).

No real conclusions as to the effect of oxLDL on 78NP induction can be drawn from this data. The effect does not appear to be readily reproducible, although general trends can be seen. Certainly oxLDL, under these conditions, appears to have no decisive effect on 78NP production in the cell types studied. It is unlikely, then, that 78NP is being induced in the viability studies with oxLDL in earlier chapters, at least not to a level at which it may affect viability.

B. CELL VIABILITY OF MONOCYTES AND MACROPHAGES WITH IFNγ

In an *in vivo* situation, cells would have to produce 78NP themselves if it were to act as a protectant in an inflammatory environment. The quantity of 78NP the three cell types are able to produce under stimulation with IFNγ has turned out to be very much lower than the concentrations that were used in the earlier chapters to protect cells against oxLDL and AAPH. Nevertheless, the fact that the 78NP is produced endogenously may mean that it is more efficient as an antioxidant.

In this section, the effect of adding 300U/mL IFNγ to cells challenged with oxLDL is examined. The first set of experiments use both monocyte cell types, and look at what happened when the IFNγ was added to the cells at around the same time as the oxLDL, and a 48 hour incubation was performed. It was found that more precise results were obtained when the IFNγ was added to wells containing RPMI 1640 ten minutes before the cells were added, to allow it to be distributed evenly around the solution. It was then more likely that all cells received equal cytokine stimulation.

No protective effect of the IFNγ was found in THP-1 cells when the analysis was carried out by MTT assay, but 300U/mL IFNγ alone caused a slight viability loss (Figure 89). A protective effect against the oxLDL was evident when the trypan blue assay was used, which was very significant at $p \leq 0.001$, although also very small (Figure 90). Again a small viability loss was caused by the IFNγ by itself.

In U937 cells, again no protective effect of IFNγ was apparent with the MTT assay, and the viability loss from the IFNγ alone was more pronounced (Figure 91). The trypan blue assay analysis results (Figure 92) mirrored those of the THP-1 cells, with a slight but very significant ($p \leq 0.001$) protective effect of IFNγ, and a cell viability loss with IFNγ only.

The protection against oxLDL shown with the trypan blue assay in monocyte cell lines may represent a delaying of apoptosis, slight enough to affect loss of membrane permeability, but not the loss of enzyme function measured by the MTT assay.

78NP may have contributed to this effect, but there is no proof of this. Other products of IFNγ stimulation may have also played a role or been completely responsible for the protection. Although no extra tryptophan was added, evidence has also been presented that RPMI 1640 media contains high enough levels, at around 25μM, for 3HAA to be made (Christen *et al.*, 1994). IFNγ might also delay apoptosis through the reduction of caspase activation and the blocking of cell proliferation (Lotem *et al.*, 1996; Xaus *et al.*, 2001). The lack of a decrease in intracellular oxidative stress levels may suggest the effect is not antioxidant-based, making a contribution by 78NP or 3HAA less likely, but this would need to be further investigated (Lotem and Sachs, 1996).

In THP-1 cells differentiated with PMA, scavenger receptor expression and activation is inhibited by IFNγ and other cytokines. This may make the cells less susceptible to oxLDL-induced cytotoxicity, if that toxicity requires uptake of the oxLDL (Hsu *et al.*, 1996). Alternatively, the cells may become more susceptible, if uptake decreases the oxLDL's toxicity (Lougheed *et al.*, 1991). Here, it was found that IFNγ did not change the viability loss due to the oxLDL in the THP-1 macrophage-like cells, as analysed with the MTT assay, but did alter cell viability alone ($p \leq 0.01$) (Figure 93).

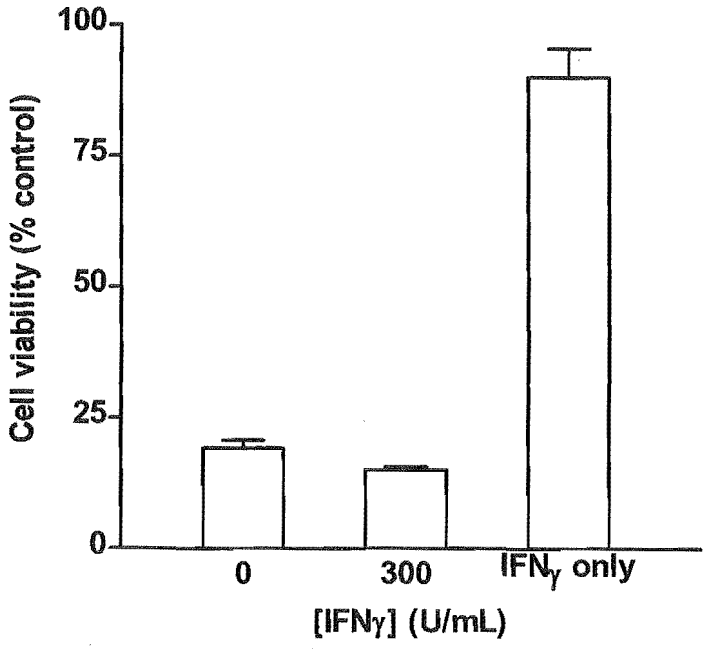

Figure 89: Effect of IFNγ on oxLDL-induced cell viability loss in THP-1 cells: MTT assay.
THP-1 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ in RPMI 1640 for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

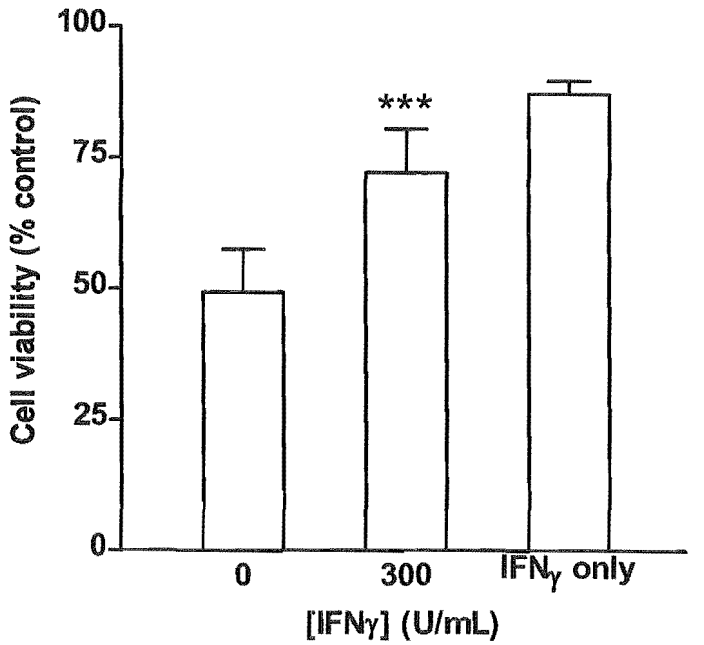

Figure 90: Effect of IFNγ on oxLDL-induced cell viability loss in THP-1 cells: trypan blue assay.
THP-1 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ in RPMI 1640 for 48 hours. A 300U/mL IFNγ-only control was included. The trypan blue assay was used, and results are expressed as mean ± SD of triplicates.

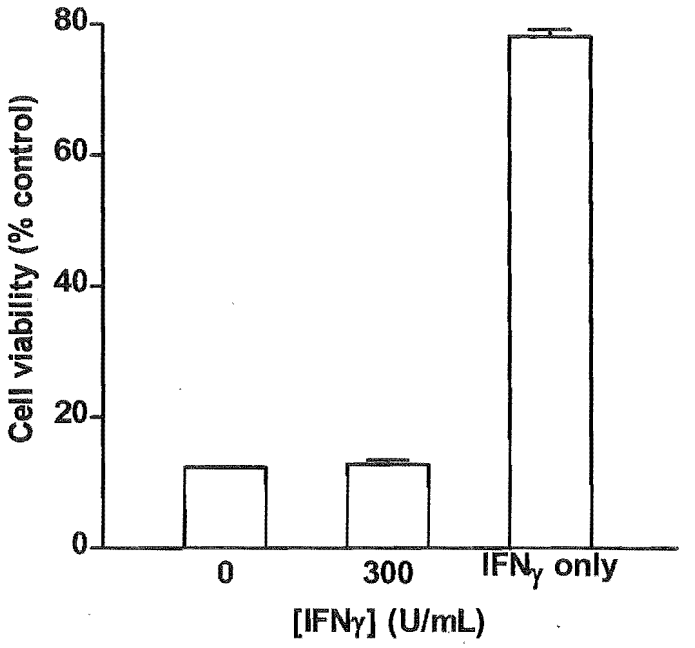

Figure 91: Effect of IFNγ on oxLDL-induced cell viability loss in U937 cells: MTT assay.
U937 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ in RPMI 1640 for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

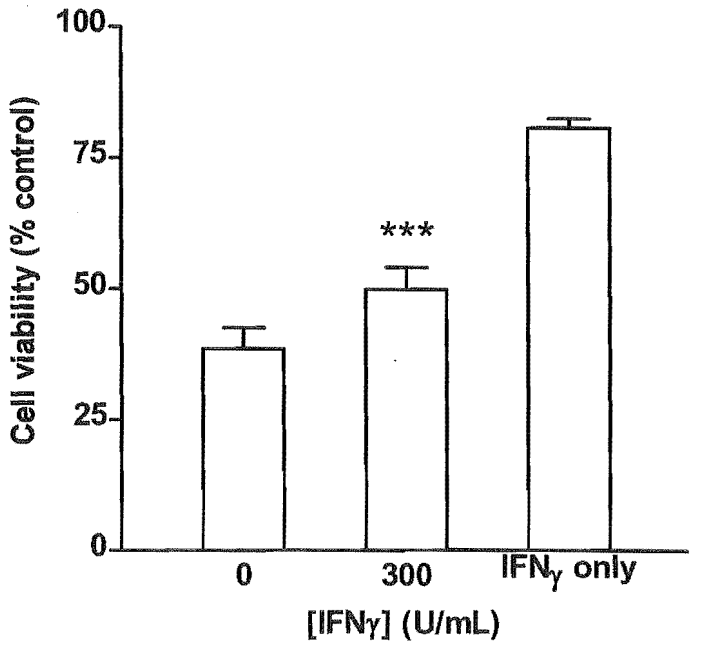

Figure 92: Effect of IFNγ on oxLDL-induced cell viability loss in U937 cells: trypan blue assay.
U937 cells (5 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ in RPMI 1640 for 48 hours. A 300U/mL IFNγ-only control was included. The trypan blue assay was used, and results are expressed as mean ± SD of triplicates.

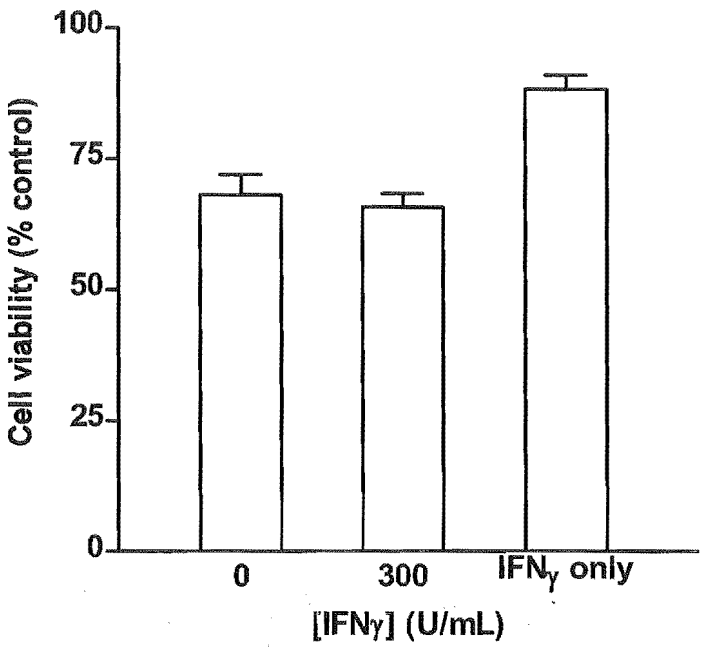

Figure 93: Effect of IFNγ on oxLDL-induced cell viability loss in THP-1 macrophage-like cells: MTT assay. THP-1 macrophage-like cells (1 x 10^5cells/mL) were incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ in RPMI 1640 for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

Perhaps a greater protective effect might be found if time for 78NP production was allowed before addition of oxLDL. Cells were incubated with IFNγ at 300U/mL in a flask for 24 hours before they were washed and aliquoted into wells for addition of oxLDL. The differentiated cells were preincubated in the same way in their wells and washed before the start of the experiment. To distinguish between the importance of the 78NP that was produced and retained inside the cells, and that which was excreted, the viability part of the experiment was performed in two ways. In the first case, the cells were washed after incubation with IFNγ, and were placed in the wells in a new RPMI 1640 solution, with fresh IFNγ. In the second case, the media used for the 24 hour incubation was reused, being plated out into the wells for the viability part of the experiment, so that any 78NP that was excreted into the medium during the incubation was still present. No new IFNγ was added, since the dose used for the incubation was still present and able to be effective. In the differentiated cells, new media was used, with fresh IFNγ added.

In THP-1 cells with new media and IFNγ, no protective effect of IFNγ was found with either MTT or trypan blue assays (Figures 94 and 95). The same result was achieved with the reused media (Figures 96 and 97). In two cases there was even a decrease in cell viability once IFNγ was also added, compared with just oxLDL. The decrease was only significant ($p \leq 0.001$) in Figure 97, THP-1 cells in reused media assayed with trypan blue.

In U937 cells the outcome was identical. No protective effect of IFNγ against oxLDL was found with either with the new or the reused media, analysed with either assay. These results are illustrated in Figures 98-101. The same result was found with the differentiated THP-1 cells, in Figure 102.

Instead of the more pronounced protective effect expected after IFNγ preincubation, in this set of experiments IFNγ does not seem to positively affect oxLDL-induced cell viability loss, even when fresh IFNγ is added. The protective function found with the trypan blue assay in Figures 90 and 92 appears to be lost when cells are preincubated with IFNγ. This implies the reduction in cell death seen in the earlier figures was not due to 78NP, as its concentration does not decrease hugely over this timeframe.

The cells may become desensitized to IFNγ over the 24 hours of preincubation, so that the changes the IFNγ causes which are seen in a retarding of cell death are reduced over time. This phenomenon has been described before. It was found that repeated stimulation with LPS caused tolerance to develop, diminishing IL-12 production in human monocyte-derived macrophages (Monteleone *et al.*, 1999). Macrophages isolated from inflammatory sites in the body are desensitized towards LPS and IFNγ, producing less TNFα. After ten days in culture, cytokine production becomes normalized (Burger *et al.*, 1999).

Alternatively, the apoptosis-causing effect of the IFNγ may become more pronounced with longer incubation with IFNγ, canceling out the protective effect. For example, preincubation with IFNγ has been shown to upregulate pro-caspases and downregulate anti-apoptotic proteins (Varela *et al.*, 2001), and this may take some time to occur to a degree where it is measurable as cell viability loss.

In conclusion, even when allowed time to build up a greater concentration of 78NP, monocytes are not able to protect themselves from oxLDL due to stimulation with IFNγ. IFNγ alone causes a loss of viability of around 20-30% relative to the untreated cells.

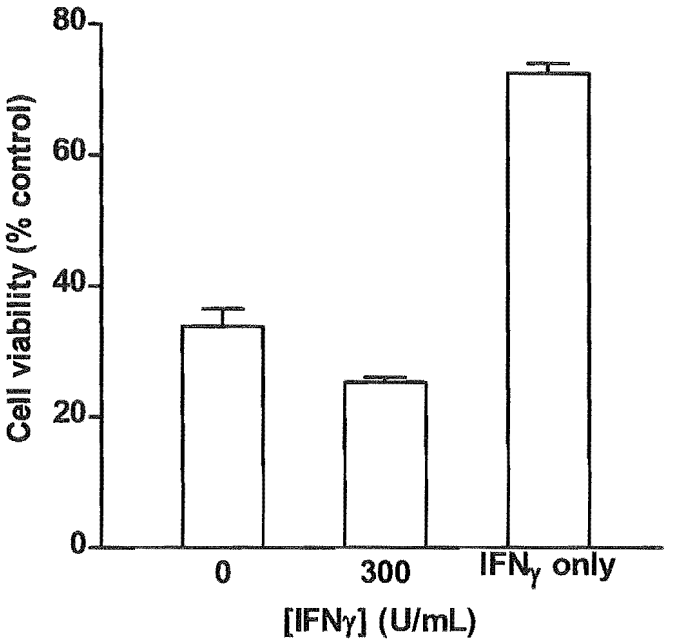

Figure 94: Effect on oxLDL-induced cell viability loss of preincubating THP-1 cells with IFNγ, and changing the media for incubation with oxLDL: MTT assay.
THP-1 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in new RPMI 1640 media (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

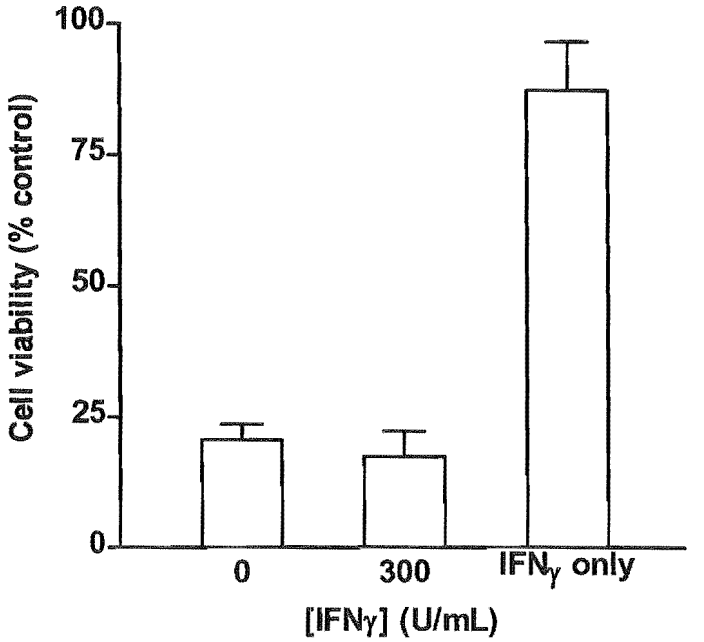

Figure 95: Effect on oxLDL-induced cell viability loss of preincubating THP-1 cells with IFNγ, and changing the media for incubation with oxLDL: Trypan blue assay.
THP-1 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in new RPMI 1640 media (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ for 48 hours. A 300U/mL IFNγ-only control was included. The trypan blue assay was used, and results are expressed as mean ± SD of triplicates.

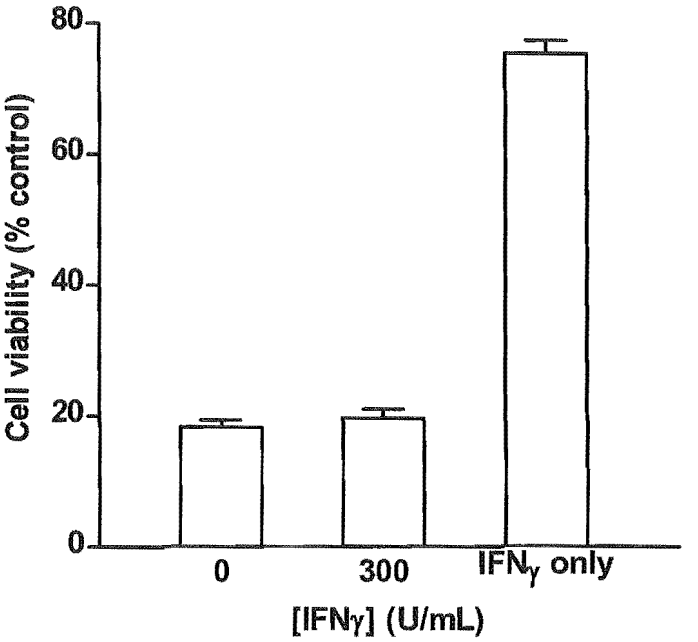

Figure 96: Effect on oxLDL-induced cell viability loss of preincubating THP-1 cells with IFNγ, and reusing the media for incubation with oxLDL: MTT assay.
THP-1 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in the RPMI 1640 media they were preincubated in (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

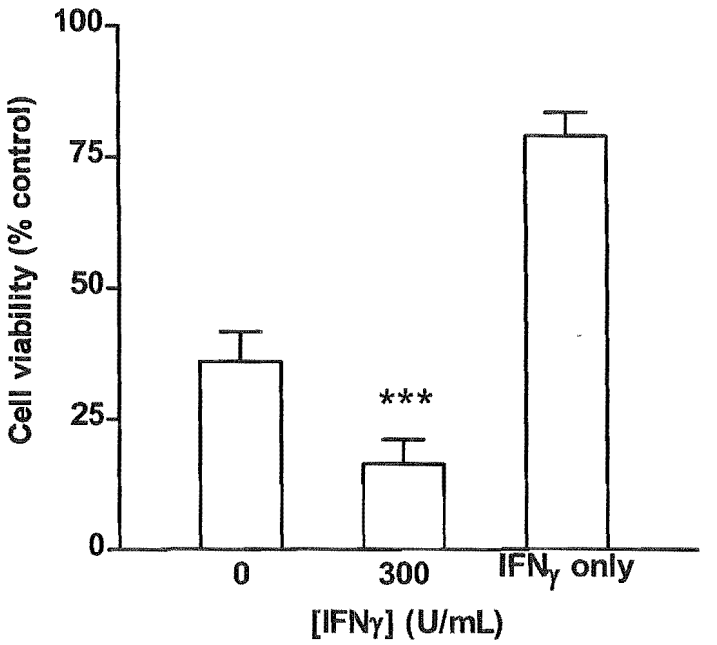

Figure 97: Effect on oxLDL-induced cell viability loss of preincubating THP-1 cells with IFNγ, and reusing the media for incubation with oxLDL: Trypan blue assay.
THP-1 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in the RPMI 1640 media they were preincubated in (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL for 48 hours. A 300U/mL IFNγ-only control was included. The trypan blue assay was used, and results are expressed as mean ± SD of triplicates.

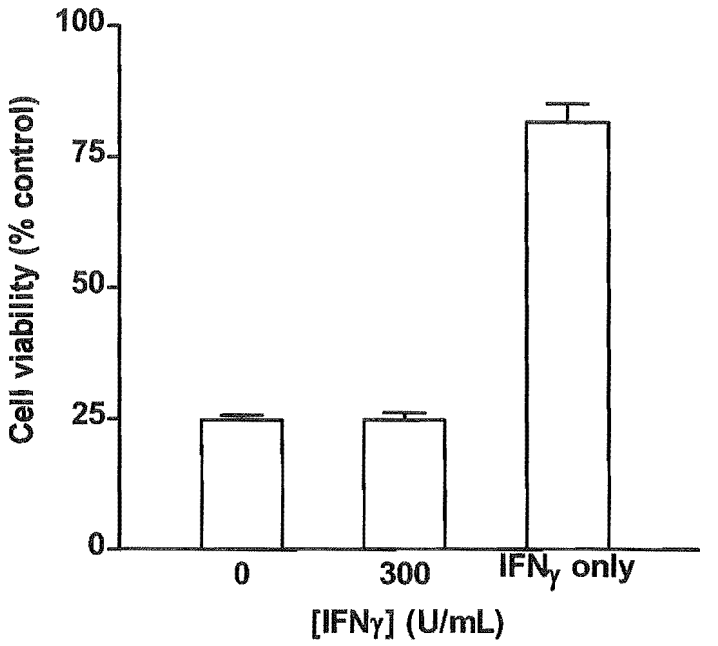

Figure 98: Effect on oxLDL-induced cell viability loss of preincubating U937 cells with IFNγ, and changing the media for incubation with oxLDL: MTT assay.
U937 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in new RPMI 1640 media (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

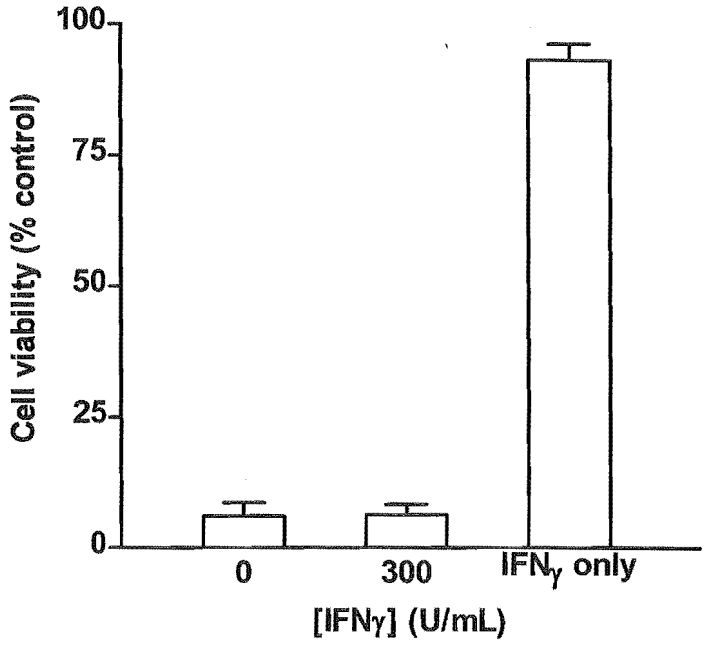

Figure 99: Effect on oxLDL-induced cell viability loss of preincubating U937 cells with IFNγ, and changing the media for incubation with oxLDL: Trypan blue assay.
U937 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in new RPMI 1640 media (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL with or without 300U/mL IFNγ for 48 hours. A 300U/mL IFNγ-only control was included. The trypan blue assay was used, and results are expressed as mean ± SD of triplicates.

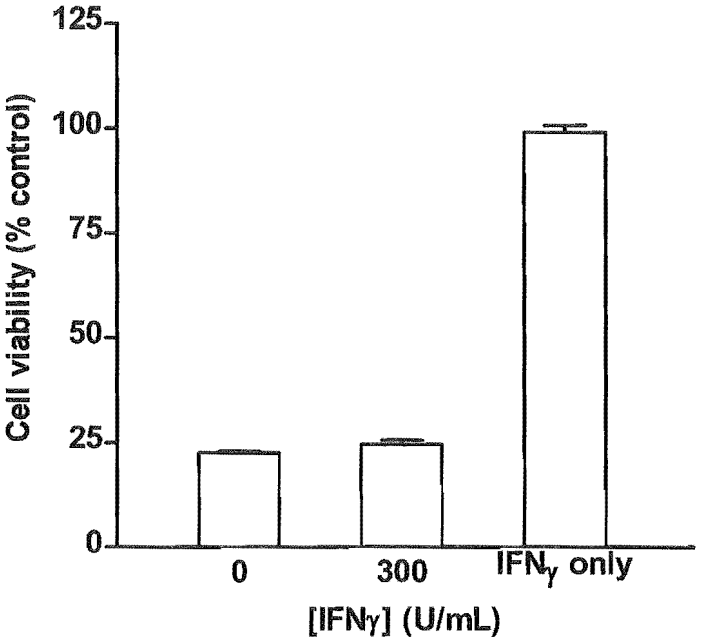

Figure 100: Effect on oxLDL-induced cell viability loss of preincubating U937 cells with IFNγ, and reusing the media for incubation with oxLDL: MTT assay.
U937 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in the RPMI 1640 media they were preincubated in (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

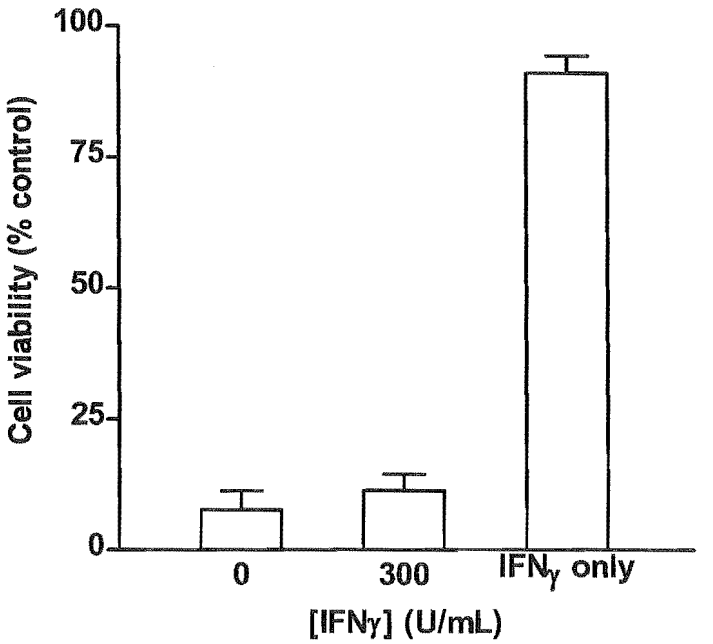

Figure 101: Effect on oxLDL-induced cell viability loss of preincubating U937 cells with IFNγ, and reusing the media for incubation with oxLDL: Trypan blue assay.
U937 cells were preincubated with 300U/mL IFNγ for 24 hours before being washed and resuspended in the RPMI 1640 media they were preincubated in (5 x 10^5cells/mL) and incubated with 1.5mg/mL oxLDL for 48 hours. A 300U/mL IFNγ-only control was included. The trypan blue assay was used, and results are expressed as mean ± SD of triplicates.

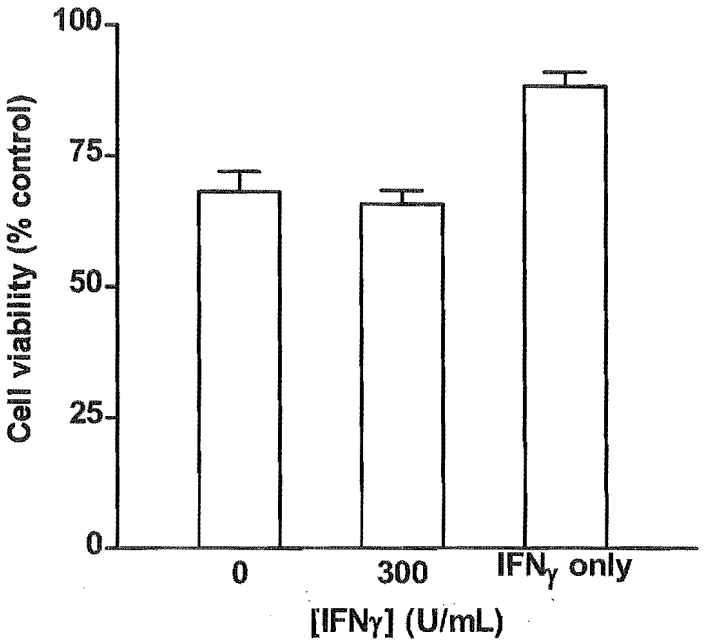

Figure 102: Effect on oxLDL-induced cell viability loss of preincubating THP-1 macrophage-like cells with IFNγ, and changing the media for incubation with oxLDL: MTT assay.
THP-1 macrophage-like cells (1 x 10^5cells/mL) were preincubated with 300U/mL IFNγ for 24 hours before being washed and incubated in new RPMI 1640 media and 1.5mg/mL oxLDL with or without 300U/mL IFNγ for 48 hours. A 300U/mL IFNγ-only control was included. The MTT assay was used, and results are expressed as mean ± SD of triplicates.

IFNγ-MEDIATED APOPTOSIS

As well as being able in some cases to reduce apoptosis, as outlined above, it has previously been reported that IFNγ may cause cell viability loss. IFNγ brought about apoptosis in human erythroid progenitor cells by downregulating their protective mechanisms and upregulating FLICE and various caspases (Dai and Krantz, 1999). IFNγ could cause apoptosis in human peripheral blood monocytes and macrophages through cell activation, with secondary necrosis following rapidly, as measured by trypan blue exclusion. No reactive oxygen species scavengers were able to protect against IFNγ, but protein kinase C was found to be activated before apoptosis (Munn *et al.*, 1995). IFNγ induced the expression of caspases -1 and -5 in U937 and THP-1 cells (Lin *et al.*, 2000) and caused apoptosis of T cells *in vitro*

via NO production by macrophages, completing a negative feedback loop (Dalton *et al.*, 2000). IFNγ could upregulate FasL in U937 cells (Sumimoto *et al.*, 1994).

IFNγ is also known to make cells more sensitive to apoptosis (Chinetti *et al.*, 1998). This appears to occur with the THP-1 cells and oxLDL (Figure 97). In HUVECs, IFNγ alone did not cause viability loss, but in combination with TNFα or LPS, it did, possibly by induction of NO production (Werner-Felmayer *et al.*, 1993). In a similar manner, IFNγ made U937 cells more sensitive to Fas and TNFα-mediated apoptosis (Matsuda *et al.*, 1991; Nonaka *et al.*, 1999), and to γ-irradiation and etoposide (Tamura *et al.*, 1996). IFNγ also sensitised THP-1 and HL-60 cells to Fas and TRAIL-mediated apoptosis. Procaspase-8 was upregulated, and Bcl-2 downregulated by the IFNγ-preincubation (Varela *et al.*, 2001).

A short investigation was carried out to find out the way in which IFNγ may be causing this viability loss in THP-1 and U937 cells. Two avenues of inquiry were examined. Thiol loss in both monocyte cell types was looked at, as a change in cellular thiol redox potential could affect viability. A small thiol loss was found after incubating THP-1 (Figure 103) or U937 cells (Figure 104) with 300U/mL or 1500U/mL IFNγ. There was about the same amount of thiol oxidation as viability loss. The thiol loss was concentration-dependent, in that more occurred with the higher IFNγ concentration, but in U937 cells the difference between the IFNγ doses was barely significant ($p \leq 0.05$), and it was not significant at all in THP-1 cells. This does not reflect the five-fold increase in IFNγ concentration. Just as with 78NP production in the earlier work, the effect of IFNγ plateaus, reaching its maximum effectiveness between 300U/mL and 1500U/mL, after which no further additions influence the effect. IFNγ receptors may be saturated at this point. The small drop in thiols, or change in intracellular thiol redox potential this indicates, may well contribute to the viability loss. The thiol drop might also be a consequence, rather than a cause, of viability loss. The two cannot be differentiated from this data.

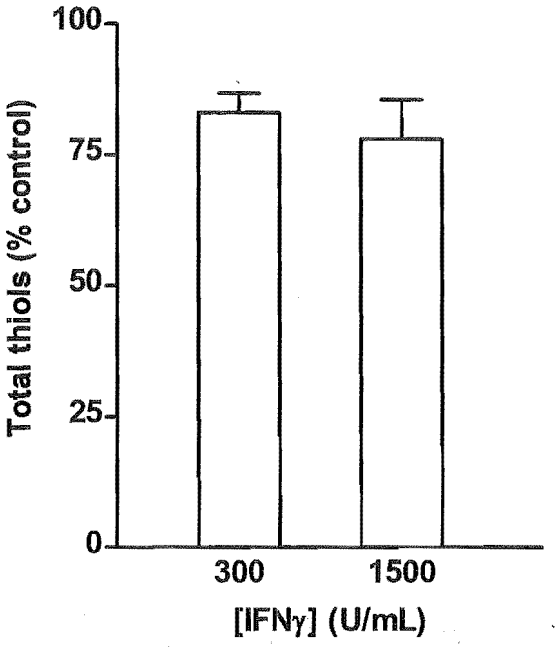

Figure 103: Effect of IFNγ on reduced thiol loss in THP-1 cells: DTNB assay.
THP-1 cells (5 x 10^5cells/mL) were incubated with two concentrations of IFNγ in RPMI 1640 for 48 hours. The analysis was done using the DTNB assay. Results are expressed as the mean ± SD of triplicates.

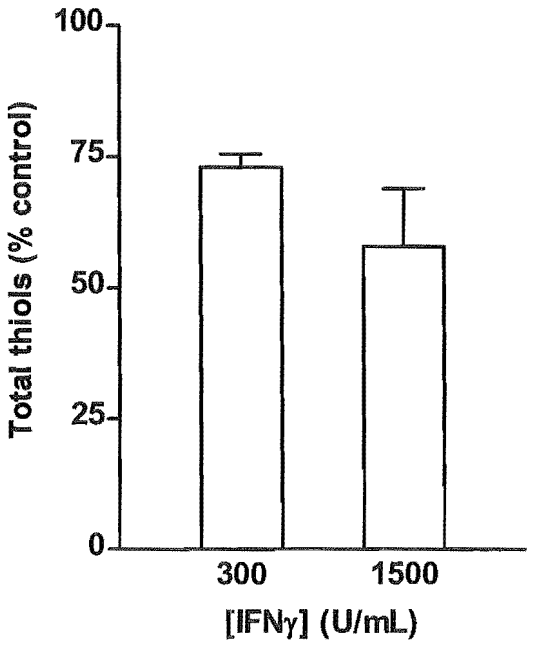

Figure 104: Effect of IFNγ on reduced thiol loss in U937 cells: DTNB assay.
U937 cells (5 x 10^5cells/mL) were incubated with two concentrations of IFNγ in RPMI 1640 for 48 hours. The analysis was done using the DTNB assay. Results are expressed as the mean ± SD of triplicates.

The other factor investigated as a possible cause of the IFNγ-mediated cell viability loss was the biological messenger nitric oxide, NO. iNOS is found in human blood monocyte-derived macrophages after stimulation with IFNγ (Reiling *et al.*, 1994). The induction can be blocked by antioxidants such as N-acetylcysteine (Pahan *et al.*, 1998).

NO's derivatives are mutagenic (Lewis *et al.*, 1995), and can be cytotoxic through the inhibition of enzymes (Mullet *et al.*, 1997; Wang *et al.*, 1997; Halliwell and Gutteridge, 1999). Part of the inhibition is due to its interactions with protein thiol groups (Messmer *et al.*, 1996). It can also nitrosylate the thiol group of glutathione (Berndji *et al.*, 1999).

Several cell types, including RAW264.7 macrophages, rat alveolar epithelial cells and human T cells, undergo apoptosis as a result of IFNγ-stimulated NO production. Hepatocytes are resistant to NO-induced apoptosis (Messmer and Bruene, 1995; Schobersberger *et al.*, 1996; Bruene *et al.*, 1997; Okada *et al.*, 1998; Dalton *et al.*, 2000; Tendler *et al.*, 2001).

U937 cells undergo p53-independent apoptosis in the presence of NO (Messmer and Bruene, 1995). However, NO can also inhibit TNF-α-mediated apoptosis in this cell type. At a late stage of apoptosis, this occurs by a cGMP-independent mechanism involving S-nitrosylation and inactivation of caspases. At an early stage, ceramide generation, which potentiates this apoptosis, is inhibited (De Nadai *et al.*, 2000).

NO's production in human macrophages, in spite of the presence of iNOS, is somewhat controversial. Around half the studies attempting to measure it do not find it, although procedures used in other papers are followed exactly (Reiling *et al.*, 1994). Studies which do detect it find that the production rate is low and rather slow. The highest reported human monocyte-macrophage level was found after stimulation of the cells with *M. avium* and TNFα. After 7 days, 211±20μM/10^6cells was achieved. This result is very much the exception, most concentrations being in the very low nanomolar range. The highest result with IFNγ was 26nmol/10^6cells over 24 hours (Albina, 1995). Using the Ca^{2+} ionophore ionomycin, values for NO production over 10 minutes with 5 x 10^5cells/mL were obtained for THP-1 cells (30.9±1.8nM), U937 cells (26.2±2.0nM) and peripheral blood monocytes (32.1±8.7nM) (King *et al.*, 1997). Apoptosis of human monocytes and macrophages by IFNγ is therefore not necessarily due to NO (Munn *et al.*, 1995).

NO concentration in THP-1 monocytes incubated with 300U/mL IFNγ for up to 48 hours is shown in Figure 105. RPMI 1640 gave a slight absorbance alone, so a reading was carried out for incubated media only at each timepoint and subtracted from the value of the cell lysate or supernatant sample. It was found that there was no further nitrite in the cell lysate, so the graphed results represent the values obtained from the supernatant.

There was no difference in NO production between cells with or without IFNγ. In both cases, the levels were much lower than those reported in the studies outlined above. NO is therefore not the cause of the low levels of cell death resulting from IFNγ in the experiments here.

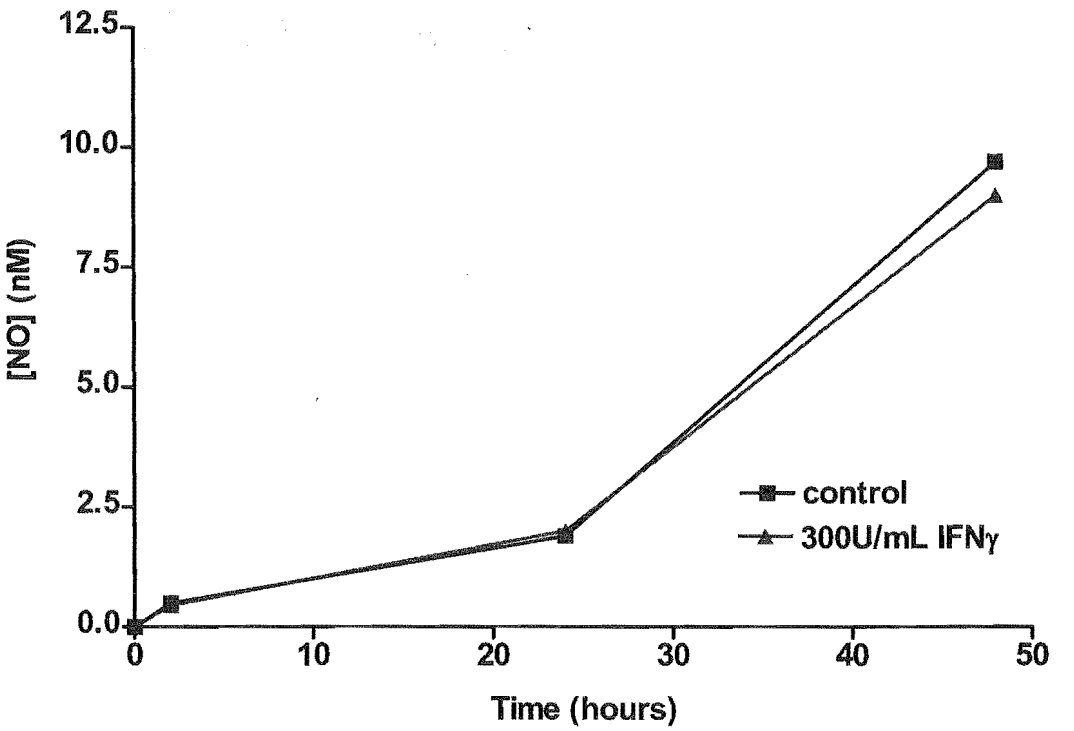

Figure 105: Production of NO in THP-1 cells with and without IFNγ.
THP-1 cells (5 x 10^5cells/mL) were incubated with or without 300U/mL IFNγ in RPMI 1640 for up to 48 hours. At certain timepoints, cells were removed, washed twice with PBS, lysed by sonication and analysed for NO production with the Griess reagent at 540nm. NO concentration was found from a standard curve constructed using sodium nitrite. Results are expressed as the mean ± SD of triplicates.

IFNγ might be causing a loss of cell viability by upregulating caspases, or downregulating antiapoptotic factors such as Bcl-2, as was reported above. The small drop in reduced thiols found in Figures 103 and 104 may be affecting the cell viability. Another possibility is that IFNγ is in fact beginning to cause a partial differentiation of the cells by 48 hours, slowing cell growth. If growth was therefore occurring faster in the untreated cells, the impression of a slight loss of viability might be given. It is unlikely though, that much growth is occurring, as the cells are deprived of the nutrients required for growth which are contained in serum.

Since there was a drop in cell viability during incubation with IFNγ, it was investigated whether 78NP could improve the levels of viability. Greater concentrations of the antioxidant induced by IFNγ could perhaps balance the deleterious effects it is creating. However, this was not found to be the case when THP-1 cells (Figure 106) and U937 cells (Figure 107) were incubated with 300U/mL IFNγ with or without 200μM 78NP. Both cell types gave the same result, showing no significant decrease in viability loss due to the addition of the 78NP.

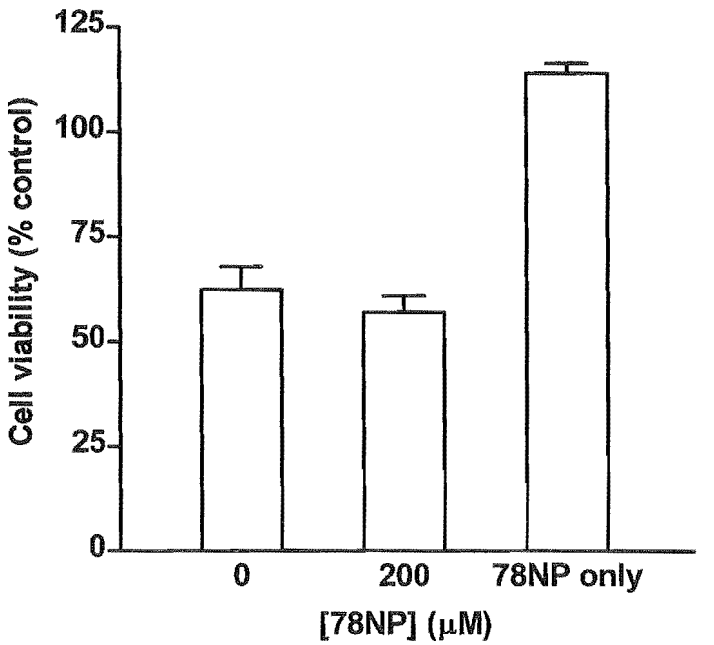

Figure 106: Effect of 78NP on IFNγ-mediated THP-1 cell viability loss: MTT assay.
THP-1 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 300U/mL IFNγ with or without 200μM 78NP for 48 hours. The MTT assay was used for analysis. A 200μM 78NP only control was included. Results are expressed as the mean ± SD of triplicates.

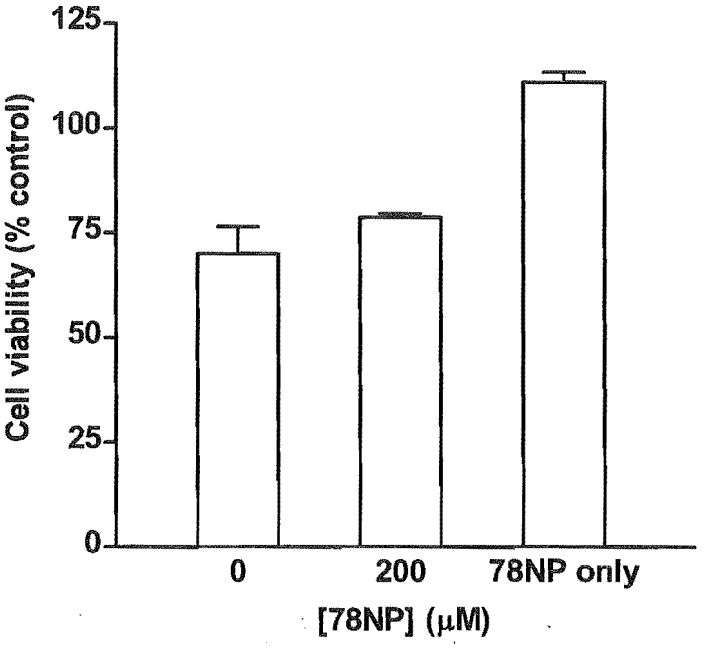

Figure 107: Effect of 78NP on IFNγ-mediated U937 cell viability loss: MTT assay.
U937 cells (5 x 10^5cells/mL) were incubated in RPMI 1640 with 300U/mL IFNγ with or without 200μM 78NP for 48 hours. The MTT assay was used for analysis. A 200μM 78NP only control was included. Results are expressed as the mean ± SD of triplicates.

This result offers no support for the idea that 78NP might reduce the cell viability decreasing effects of IFNγ. It is possible that the 78NP produced may indeed be working this way, but at a location the exogenously added 78NP cannot reach, or else in such a way that it is already carrying out its maximum possible protective effect, and no extra 78NP will be able to affect this. This could be tested by knocking out an enzyme responsible for 78NP, but this would be complicated as the enzymes specific for this pathway, for example GTP cyclohydrolase I, lead to other pteridine products such as 5,6,7,8-tetrahydrobiopterin.

Higher levels of 78NP might be produced *in vivo*. Direct cell-to-cell contact may favour accumulation of higher concentrations in the microenvironments of cells, which could be further augmented in concert with other cytokines acting upon cells with IFNγ, such as IL-1 and TNFα (Wirleitner *et al.*, 2001).

C. LEVELS OF 78NP AND NEOPTERIN *IN VIVO*

Inflammatory material was gathered from consenting patients at the DSA Unit in the Radiography department of Christchurch Hospital. Samples of other fluids, such as plasma, could not be obtained from patients, as it was inappropriate to subject such ill patients to further invasive sampling.

All pus samples were found to contain pterin. A comparison of the levels of pterin and 78NP in each revealed that inflammatory material, as expected, contained much more pterin than reported in serum.

In several cases, the amount of 78NP in pus appeared to be negative. The 78NP concentration was calculated by measuring pterin fluorescence with and without acidic iodide oxidation of the sample. Oxidation was used for total pterin, to show both 78NP and neopterin. Only neopterin will show up before oxidation. Where 78NP seemed negative, more fluorescence was found in the samples before oxidation than afterwards, total neopterin having been lost. This may be a result of reaction with other substances present in the pus, which may only be present after reaction with acidic iodide. The addition of acidic iodide greatly changed the size of every peak in pus samples, but to varying extents in each sample, suggesting different constituents.

The level of pterin, although measurable, was not at all close to the range required in experiments in earlier chapters to protect cells from viability loss and apoptosis caused by AAPH or oxLDL. Higher 78NP concentrations might be necessary to see an effect in cells in this type of experiment, since the dose is only added once, whereas *in vivo* high levels of neopterin persist in body fluids of patients. It could be argued that the 78NP may be more concentrated in the microenvironment surrounding the cell that produces it (Gieseg *et al.*, 2000). Levels of 78NP may also have been higher than they appeared if 78NP had oxidised to 78XP, which was not measured. Since the development of an inflammatory, or particularly an atherosclerotic site, is a far longer process than any of the experiments undertaken here, a lower concentration of 78NP might be sufficient to protect the cell. It seems unlikely, however, that these situations will be able to be verified.

Table 7: Pterin measurements in pus.
Inflammatory material was brought to the laboratory on ice and frozen at –80°C until use. Samples were prepared by addition of nanopure water, BHT and EDTA under aseptic conditions, followed by homogenization for one minute. Samples were analysed immediately by the HPLC pterin method with and without acidic iodine solution, and protein determinations were carried out for each sample. Results are expressed as the mean ± SD of triplicates.

Patient code	Sex	Age	Origin of pus	Total pterin (pmol/mg protein)	Total pterin (nM)	%78NP	[78NP] (nM)
DE	F	43	Gallbladder fossa	5.7±1.2	46.9±4.0	-56.1	-25.8±5.9
NM	M	61	Peripancreatic	19.1±3.0	419.0±5.0	-17.9	-74.9±9.7
PM	M	44	RHS plural space	6.9±0.3	365.5±11.0	-9.6	-35.1±17.6
MH	F	49	Paracoli region	16.5±2.3	517.2±18.0	45.9	237.1±0.1
RB	M	51	Gallbladder fossa	8.7±1.6	67.2±3.0	-22.2	-15.1±3.6
NH	F	69	Anterior abdominal wal	1.7±1.2	68.0±4.0	-16.2	-11.0±5.0
LM	F	34	Pelvis	6.8±0.6	496.6±46.0	-3.82	-19.0±0.02
VL	F	89	Gallbladder	10.4±0.4	692.0±26.0	13.5	93.4±0.003
HG10	F	62	Subphrenic	16.3±0.9	543.6±29.0	11.6	63.4±0.02
DJ	M	68	RHS flank	10.2±0.3	692.0±21.0	77.0	533.7±0.01
NP	F	41	RHS anterior plural space	9.8±1.2	580.0±71.0	41.8	242.2±0.04
AC	F	24	Fistula/abcess RHS	15.6±05	651.6±19.0	12.1	78.7±0.01
MG	F	74	Pelvis	16.7±1.9	860.0±99.0	52.3	449.8±0.1
BC	F	64	Pelvis	19.9±3.5	889.0±157.0	35.3	314.1±0.09
HG8	F	62	Subphrenic	17.0±1.6	639.0±61.0	37.4	238.9±0.05
PE	M	53	Lower abdomen	14.4±1.1	1281.0±96.0	15.7	200.7±0.04
CB	M	89	Abdominal cavity	9.6±1.9	96.0±2.0	-15.0	-14.4±2.4
GM	M	48	Pararectal, lower pelvic	7.8±0.6	304.0±22.0	43.1	131.2±0.01
WT	M	73	Paracoli, lower pelvic	16.9±0.8	403.2±20.0	4.3	17.3±0.004

CORRELATIONS

An examination of the various pterin measurements grouped according to sex suggested that females may have a slightly higher concentration of pterin in their inflammatory material, and that this material might contain a greater proportion of 78NP, however these correlations were not statistically significant (Table 8).

Table 8: Comparison of pterin measurements between male and female patients.

	All (Mean±SD)	Female (Mean±SD)	Male (Mean±SD)
Total pterin (pmol/mg)	12.11±5.20	12.40±5.84	11.70±4.52
% 78NP	29.56±22.07	31.67±20.37	26.66±25.35
[78NP] (nM)	178.83±159.60	196.68±155.00	154.29±173.16

Possible correlations between pterin and 78NP concentrations, pterin or 78NP and protein concentration, and the age of the patient, pterin, 78NP or protein were also examined, using the Prism program. Correlations were assessed using the Pearson correlation co-efficient, r, r^2, and two-tailed p-values.

Significant correlations were only found for the comparison of 78NP and pterin. When measured in pmol/mg, r^2 was 0.4221, Pearson r was 0.6497 and p was 0.0026 (** in the system used on the earlier graphs). When the pterin value was expressed as μM, and the 78NP value as nM, r^2 was 0.4042, Pearson r was 0.6358 and p was 0.0034 (also **). This means that a patient with more pterin overall is likely to also have more 78NP. The correlation is not at the greatest level of significance, suggesting that other factors, such as the extent and type of inflammatory reaction, for example, will impact on how much 78NP is oxidised in the site.

OTHER PARAMETERS

The same inflammatory material was also used for measurements of levels of vitamin E, as another representative antioxidant, and two markers of protein oxidation, PB-DOPA

and dityrosine, by other members of the laboratory (Table 9) (Gieseg *et al.*, 1993; Gieseg *et al.*, 2000; Hawkins and Davies, 2001).

Abstraction of hydrogen from tyrosine's hydroxyl group by radicals such as hydroxyl, alkoxyl or peroxyl radicals gives the phenoxyl radical TyrO·, which often crosslinks to give dityrosine. This has been found in atherosclerotic lesions (Halliwell and Gutteridge, 1999; Hawkins and Davies, 2001). It can be formed from reactions caused by myeloperoxidase, or from a metal-catalysed Fenton system *in vitro* (Huggins *et al.*, 1993).

The hydroxide 3,4-dihydroxyphenylalanine (DOPA) is formed through the reaction of a hydroxyl radical with the aromatic ring of tyrosine, followed by addition of oxygen and elimination of the hydroperoxide (Hawkins and Davies, 2001).

It was hoped that correlations would be found among the different pterin measurements and vitamin E, PB-DOPA and dityrosine, however this was largely not the case. A significant positive correlation was found between the concentration of total pterin and vitamin E (r^2=0.2283, Pearson r=0.4778, p=0.0385, *), and 78NP and vitamin E (r^2=0.3751, Pearson r=0.6125, p=0.0069, **). This may reflect the oxidative conditions of the particular inflammatory site the material was drawn from. The two antioxidants might be 'used up' at a similar pace, or the amount of vitamin E available might influence the level of 78NP production.

The lack of correlation between the two antioxidants and the two markers of oxidation allow no conclusions to be drawn about pterins or vitamin E acting as antioxidants *in vivo* to suppress oxidation resulting in PB-DOPA or dityrosine.

Highly significant positive correlations were found between the amount of protein found in the inflammatory material, and the amount of dityrosine or PB-DOPA measured. Both are protein oxidation products, so this would be expected. In the case of protein compared to PB-DOPA, r^2 was 0.8216, Pearson r was 0.9064 and $p < 0.0001$ (***). For dityrosine and protein, r^2 was 0.7177, Pearson r was 0.8472 and $p < 0.0001$ (***).

Table 9: Vitamin E, PB-DOPA and dityrosine measured in pus. These were determined by HPLC (Gieseg *et al.*, 1993; Gieseg *et al.*, 2000). The results are presented as mean ± SEM.

Patient code	Vitamin E (pmol/mg)	PB-DOPA (pmol/mg)	Dityrosine (pmol/mg)
DE	465.88±29.15	109.68±17.17	0.00±0.00
NM	397.26±19.45	225.31±26.94	12.15±1.97
PM	421.47±8.03	86.58±7.12	5.04±0.18
MH	1009.25±57.51	166.59±24.86	154.13±5.06
RB	310.21±17.82	104.47±6.58	0.00±0.00
NH	12.49±0.089	409.52±18.16	81.46±12.87
LM	290.05±19.74	70.60±1.56	2.28±0.23
VL	240.34±25.85	92.31±10.22	69.40±12.69
HG10	1148.12±28.31	75.12±3.71	3.65±0.24
DJ	79.09±6.04	262.54±20.53	1.96±0.32
NP	252.29±3.85	523.52±46.39	1.48±0.15
AC	714.04±15.78	115.09±10.28	1.20±0.072
MG	542.84±4.97	54.13±2.62	7.99±0.60
BC	252.91±16.41	580.51±21.73	1.93±0.18
HG8	1056.53±22.24	49.68±3.31	7.79±0.37
PE	337.43±7.72	297.47±25.87	4.40±0.39
CB	439.34±19.91	288.43±38.38	3.05±0.17
GM	241.27±17.22	333.20±42.70	23.94±1.06
WT	1470.37±57.52	203.77±31.30	6.93±0.64

SUMMARY

All three cell types tested produced 78NP under stimulation from IFNγ. A higher level was produced with a greater IFNγ concentration, but the increase was not proportional to the increase in IFNγ concentration. THP-1 macrophage-like cells produced the most 78NP relative to cell number and protein concentration, and U937 monocyte cells generated more than THP-1 monocytes. The maximum amount produced was a thousand-fold less than the concentrations used in viability assays to protect U937 cells. All three cell types showed the same trends over time, with an increase until 24 hours, after which the 78NP concentration tailed off.

IFNγ incubated with cells could not protect U937 monocytes, THP-1 monocytes or THP-1 macrophage-like cells from cell viability loss caused by oxLDL, even if preincubated with the cells to allow time for 78NP to be produced. It caused a small loss of viability itself, which was not due to NO production, but may relate to a slight loss of reduced thiols, caspase upregulation or downregulation of anti-apoptotic proteins. 78NP at 200μM was unable to protect the cells from IFNγ-mediated cell viability loss.

78NP and neopterin levels measured in inflammatory material were higher than those found in serum. However, the levels were still a thousand-fold lower than those required to protect cells from oxLDL or AAPH-mediated viability loss in *in vitro* experiments.

SUMMARY AND CONCLUSIONS

The primary focus of this study was to investigate whether 7,8-dihydroneopterin could protect monocytes and macrophages from the cytotoxic effects of biologically relevant concentrations of oxLDL *in vitro*. It has been hypothesised that 78NP is synthesised *in vivo* as a macrophage protectant and thus may be important in plaque formation and stability during atherosclerosis. During this research, a second focus developed, describing the process of cell death induced by the oxLDL and how it differed between the two monocyte cell types examined.

EFFECT OF OXLDL ON CELLS

Both THP-1 and U937 monocytes were found to undergo concentration-dependent viability loss in the presence of oxLDL (Figures 29 and 34), which occurred to a similar degree in each cell type. The oxLDL concentrations tested were comparable with those found *in vivo* in the intima. However, while THP-1 cells showed a gradual loss of reduced thiols, similar to the level of cell viability loss, U937 cell thiols were suddenly and completely depleted with 1.5mg/mL oxLDL (Figures 39 and 40). A critical point for this oxLDL concentration was reached after six hours of incubation, after which time it appeared that maintenance of reduced thiols was not possible (Figure 42). Potential mechanisms of glutathione loss reported previously in U937 cells include efflux, oxidation and downregulation of synthesis. A small proportion of the reduced thiols may also be lost from proteins, including important enzymes and transcription factors such as NFκB.

The difference between the two monocyte cell types may relate to the way the oxLDL interacts with the cells. The monocytes are unlikely to have scavenger receptors (Akeson *et al.*, 1991A), although these may be induced during incubation with oxLDL (Lei *et al.*, 2002). U937 cells have higher CD36 levels than THP-1 cells (Nguyen-Khoa *et al.*, 1999) and THP-1 cells a greater ability to phagocytose than U937 cells (Sundström and Nilsson, 1976). However, the differing loss of caspase activity in each cell type during the incubation of cell lysates with oxLDL (Figure 72) suggested that oxLDL's access to the cell was not a factor.

The cell type difference is more likely to be part of the oxidative response to oxLDL-initiated stress. The U937 cell thiol response may be specific to oxLDL, as the sudden drop in thiols was not observed with AAPH treatment. The two cell types have different antioxidant profiles: U937 cells have less GSH, thioredoxin, thioredoxin reductase and ceruloplasmin and more MnSOD than THP-1 cells (Mazumder *et al.*, 1997; Ferret *et al.*, 2000), which may result in the cell types being differently equipped to deal with various stresses. The cell types may also have different signalling pathways altered by oxLDL. U937 cells are reported to have no PPARγ (Inoue *et al.*, 2001), which has an anti-inflammatory effect in cells during longer incubations with oxLDL (Jang *et al.*, 1999). This may result in a greater level of intracellular oxidative stress over the incubation time and a more extensive loss of reduced thiols.

The loss of thiols in U937 cells may be important for the induction of apoptosis during incubation with oxLDL. A lowering of thiol levels has been suggested to be essential for apoptosis in some cell types (Lizard *et al.*, 1998). In U937 cells, the loss resulted in the inactivation of caspase enzymes, which have a catalytic cysteine residue (Boggs *et al.*, 1998). A comparison of the loss of cell viability, thiols and caspase activity in U937 cells over time (Figure 71) showed that thiols and caspase activity decreased in parallel, whereas loss in viability was slower. Hydrogen peroxide, protein hydroperoxides, NO, selenium and zinc ions have been reported to inhibit activated caspases in a manner reversible by DTT, demonstrating thiol-dependence (Hampton and Orrenius, 1997; Stennicke and Salveson, 1997; Boggs *et al.*, 1998; Park *et al.*, 2000; Borutaite and Brown, 2001; Hampton *et al.*, 2002A; Hampton *et al.*, 2002B). OxLDL was shown to lower caspase activity in cell lysates over ten minutes (Figure 72) to a much greater extent in U937 cells than in THP-1 cells, in line with the overall effect of oxLDL on reduced thiols in these cell types. This result emphasises the specific nature of this reponse in the U937 cell type for oxLDL, as other studies have shown that U937 cells can have active caspase enzymes (Galán *et al.*, 2001).

Caspases were activated in a much more conventional fashion in THP-1 cells, which did not undergo the same extensive loss of reduced thiols. Caspase activity increased over time with increasing concentrations of oxLDL and decreased again as secondary necrosis began (Figure 67).

An alternative mechanism of apoptosis is required by the U937 cells with oxLDL. This may involve other enzymes, such as cathespins and DNases, which might be released from the lysosome if oxLDL were to accumulate inside it (Brunk *et al.*, 1997; Borner and Monney, 1999). Oxidative stress might itself be an important part of the apoptotic mechanism. Loss of reduced thiols is a passive, indirect form of oxidative stress (Ghibelli *et al.*, 1998). A lowering of glutathione levels may open the mitochondrial pore, allowing apoptosis to proceed by this route (Lizard *et al.*, 1998).

In other respects, oxLDL induced similar forms of apoptosis in U937 and THP-1 monocytes. The Hoechst 33342 stain showed both cell types to have typical apoptotic nuclear morphology, including condensation, pyknosis and fragmentation. Higher concentrations of oxLDL induced primary and secondary necrosis as well as apoptosis over 24 hours.

The Annexin V/propidium iodide assay looked at phosphatidylserine exposure on the surface of the cells. Both cell types appeared to undergo apoptosis, with THP-1 cells moving more swiftly towards (secondary) necrosis. The process would not have been caspase-dependent in U937 cells (Figures 73-78).

To confirm that apoptosis is taking place, the findings of all three assays need to be combined. From this it appears that apoptosis is likely to be occurring in both cell types, but on a continuum scale from apoptosis to necrosis, THP-1 cells undergo a more conventional form of apoptosis, whereas the U937 cells' apoptosis is more towards the centre of the scale.

PMA-differentiated THP-1 macrophage-like cells were resistant to cell viability loss with AAPH and oxLDL, even at high concentrations and over long periods of time (Figures 96, 97, 100-102). This may be due to the changes in cell metabolism that occur as a result of differentiation. For example, lipid metabolism genes and the scavenger receptor are activated (Kritharides *et al.*, 1998; Liao *et al.*, 2000), which would result in more degradation of oxLDL and less toxicity. Macrophage secondary products are also generated (Via *et al.*, 1989) and the cells'antioxidant profile changes, to include more GSH, thioredoxin, thioredoxin reductase and SOD (Gotoh *et al.*, 1993; Ferret *et al.*, 2000). PMA-induced changes also counteract some of those mediated by oxLDL. PPARγ, TNFα and PKC, the expression of which oxLDL incubation increases in monocytes, will decrease as a result of incubation with PMA (Schwende, 1996; Girona *et al.*, 1997; Jang *et al.*, 1999).

EFFECT OF 78NP ON CELLS

78NP was tested as an antioxidant or protectant in THP-1 and U937 monocytes and THP-1 macrophage-like cells with two inducers of cell viability loss: AAPH, which produces peroxyl radicals and oxLDL, thought to be the main source of cell damage and death in the plaque. The contrasts in the effect of 78NP on the THP-1 and U937 cell types allowed conclusions to be drawn regarding the nature of cellular protection by 78NP.

It has previously been shown in chemical systems that 78NP is able to scavenge a wide range of radicals very efficiently, including peroxyl radicals (Duggan *et al.*, 2002). Therefore AAPH was a logical first step in testing the response of cells to 78NP. 78NP prevented loss of cell viability and lowered reduced thiol loss in U937 cells in RPMI 1640 and EBSS media, and slightly lowered THP-1 cell viability loss in the EBSS medium only (Figures 13, 14, 19-22, 25, 26). It had no effect on THP-1 macrophage-like cells (Figure 98).

78NP has also been reported to protect cells and cellular membranes from oxidative stimuli. 78NP shielded red blood cells from haemolysis initiated by AAPH, hydrogen peroxide and HOCl (Gieseg *et al.*, 2000B) and could partially protect U937 cells from hydrogen peroxide, HOCl and Fe-mediated damage (Gieseg *et al.*, 2001). In some situations, such as when it was at millimolar concentrations, 78NP has been reported to act as a pro-oxidant or pro-apoptotic factor (Schobersberger *et al.*, 1996; Oettl 2000B).

The hypothesis was that these previously reported properties would allow 78NP to protect cells against oxLDL. 78NP reduced loss of cell viability and reduced thiols in U937 cells and had no effect on THP-1 monocyte or macrophage-like cells (Figures 32, 37, 44, 45, 103). It is possible that antioxidant activities were responsible for the protection, as the lowering of thiol loss in U937 cells appeared crucial.

The studies using AAPH led to the conclusion that while 78NP could scavenge peroxyl radicals in cell solutions, scavenging may not be the only mechanism protecting cells from viability loss. The level of protection against cell viability loss provided by scavenging was shown by the small amount of protection provided to THP-1 cells in EBSS media against AAPH. In U937 cells, lowering of thiol loss and perhaps other mechanisms also contributed, resulting in a more effective protection against cell viability overall.

The main mechanism of 78NP's protection in U937 cells may therefore be redox modulation. Lowering the loss of reduced thiols has been shown to improve U937 cell viability in previous studies (Lizard *et al.*, 1998; Nardini *et al.*, 1998). 78NP may, directly or indirectly, be able to prevent loss of protein and non-protein thiols. It was able to diminish caspase inactivation caused by oxLDL in U937 cells, raising caspase activation levels back to those of the untreated cells (Figure 70).

78NP may lower U937 cell thiol loss by preventing the thiols from becoming oxidised, by either decreasing the cell's signal or means of oxidising the thiols. It might also enhance thiol synthesis, so that those oxidised could be replaced, or decrease efflux of thiols from the cell. However, 78NP cannot regenerate reduced thiols after oxidation on BSA, making this mechanism less likely (Platt, 2002).

The effect of time of 78NP addition to an oxLDL assay system revealed details of the mechanisms of both 78NP and oxLDL. 78NP had the same protective effect in U937 cells when added to cells two hours before oxLDL as when it was added two hours afterwards. It still had some protective effect if added six hours after the oxLDL, but from 12 hours, no protective capacity was evident. Twelve hours was also the time at which the oxLDL had first caused the maximum amount of thiol loss, and at which the process resulting in the maximum amount of cell viability loss had irreversibly begun. Commitment to cell death must have occurred by this point. The effect of the time of addition of 78NP therefore depended on the timeline of effects caused by the oxidant (Figures 62-66).

78NP may also affect cell signalling. Studies have shown that 78NP can increase cGMP and Ca^{2+} and that neopterin can activate NFκB (Woell *et al.*, 1993; Hoffman *et al.*, 1996; Murr *et al.*, 1999). Neopterin is unlikely to play an important role in the studies here, as most of the 78NP was found to oxidise to 78XP rather than neopterin (Figure 47).

Importantly, 200μM 78NP or neopterin did not increase caspase activation in THP-1 cells (Figure 68), contrary to previously reported findings in other cell types (Baier-Bitterlich *et al.*, 1995; Schobersberger *et al.*, 1996).

In contrast to those results, 78NP appeared to reduce the appearance of apoptotic features in U937 cells as measured by Hoechst 33342 staining and the Annexin V/propidium iodide assay, and continued to have no effect on THP-1 cells (Figures 76 and 78). The nature of the effect on U937 cells in these two assays suggests 78NP is delaying the apoptotic

process. Phosphatidylserine exposure itself was not reduced at the 48-hour timepoint, but the number of cells reaching necrosis or entering apoptosis was decreased. The results with the Hoechst 33342 stain appeared more absolute, the oxLDL-treated cells retaining the appearance of untreated cells. As the nuclear morphological changes viewed with this assay are late apoptotic changes, a delay in the apoptotic process would ensure they had not occurred by the 24-hour point at which they were measured.

The difference in cell types may relate to the extra differentiation of the THP-1 monocyte and macrophage-like cell type. They may interact with 78NP differently, as THP-1 cells are better able to phagocytose than U937 cells, and have different cell membrane receptors (Tsuchiya *et al.*, 1980). 78NP was able to interact with both THP-1 and U937 monocyte cell types (up to 400nM), although to a different degree with each over time. The actual amount of 78NP which became associated with the cells was not itself enough to protect cells, so it is difficult to determine if this interaction was important for the survival of the cells (Figures 49-51).

The cell types' different reactions to oxidative stress (as discussed above) may mean 78NP can have a protective effect in only one cell type when they are incubated with cell death mediators such as oxLDL. The idea is supported by the fact that THP-1 cells have not yet been shown to be protected against oxLDL-induced oxidative stress by any antioxidant. The difference in cellular oxidative stress may not be so great without the stressor, as 78NP controls were able to increase THP-1 cell viability.

These studies showed that 78NP is able to act as an antioxidant or protectant for cells against oxidative stress and the reactive oxygen species of oxLDL under some conditions. It can delay or reduce apoptosis and the following secondary necrosis.

PROTECTION OF CELLS BY 78NP IN THE ATHEROSCLEROTIC PLAQUE

In Chapter 6, it was established that although all three cell types produced 78NP as a result of stimulation with IFNγ (Table 5), the levels were not high enough to protect the cells against oxLDL or IFNγ-mediated cell death (Figures 106-119, 123-124). This may or may not reflect the *in vivo* situation. The presence of a more complex environment of cytokines

and cell types may increase, as well as decrease, the quantity of 78NP generated. 78NP and neopterin levels measured in inflammatory material were higher than those reported in serum (Table 7), but were still lower than those required to protect cells from oxLDL or AAPH-mediated viability loss.

Higher levels of exogenous 78NP were able to protect some monocyte cell types in some situations, suggesting the hypothesis that 78NP might protect cells from cell death *in vivo* may be possible. 78NP, however, could not protect all monocyte cell types under all the conditions that were tested and did not protect macrophage-like cells, reinforcing the theory that results cannot be extrapolated to other cell types. 78NP was not active as a protectant at levels generated by cells treated with IFNγ, or found *in vivo*. The evidence from these model systems does not support the hypothesis that 78NP would have a protective effect on cells in the atherosclerotic plaque.

FURTHER RESEARCH

The investigations presented in this thesis suggest a variety of directions for future studies. *In vitro*, studies using 78NP may provide interesting and useful information, possibly helping to elucidate cells' responses to oxidative stress and their pathways of apoptosis.

The THP-1 and U937 monocyte systems are useful for studying the ways in which oxLDL can affect the cell in terms of oxidative stress, due to the differences in their reduced thiol loss, an area which could be studied further. The mechanism of thiol loss in each cell type, and the identification of the types of thiols lost as protein or glutathione, would provide some interesting information about the effect of oxLDL on cellular thiols.

These experiments would also allow some evaluation of the hypothesis that a major difference between the THP-1 and U937 cell types is the form and modulation of their intracellular redox state. This could be substantiated by measurement of intracellular oxidative stress, such as the level of superoxide, and intracellular antioxidants other than glutathione, including thioredoxin, SOD and catalase. The effectiveness of these antioxidants under different conditions could be examined after exposure of the cells to other types of oxidants and measurement of other types of oxidative damage, such as oxidised products of DNA, or levels of nitrated proteins after peroxynitrite treatment. This would enable a profile

to be built for each cell type outlining how they respond to a variety of conditions. Redox responses could also be examined in terms of how important they are to each cell types' apoptotic pathways. For example, cytochrome c released from mitochondria and Bcl-2 activity could be investigated, and the possible regulation of various features in U937 cells, such as phosphatidylserine exposure, which are often under caspase control, by redox modulation could be examined.

The differences in the two monocyte cell types' apoptotic mechanisms with oxLDL could be further studied to provide general information about possible apoptotic pathways. The effect of oxLDL on the lysosome or mitochondria of each cell type, and the release of proteolytic enzymes, could be investigated. Also, the different mechanisms of phosphatidylserine exposure may be of interest. The flipping of phosphatidylserine may be caspase-dependent in THP-1 monocytes and oxidative stress-dependent in U937 monocytes. Modulation of apoptotic pathways by oxidative stress, through Bcl-2 or ceramide for example, may replace caspase regulation of events.

The interaction between reduced thiols and caspase enzymes is one that could be further explored. The study using cell lysates and oxLDL, examining loss of caspase activity over time, could be extended. A more pronounced effect on caspases might be expected over a greater timeframe. It might also be interesting to add DTT after the oxLDL, to investigate whether the reduction in caspase activity could be reversed. A solution of purified caspase enzyme(s) could be used to test whether the effect of the oxLDL is direct or via effects on upstream molecules. Fractions of oxLDL could be added to this or cell lysates to elucidate which component of oxLDL is most important for carrying out this function.

In continuing to examine the development of the atherosclerotic plaque, 78NP should be tested as a protectant for human monocyte-macrophages and foam cells, and also a mixture of cell types, such human monocyte-macrophages and T cells, or human monocyte-macrophages and smooth muscle cells. This may provide a model more relevant to the *in vivo* situation, although the quality of the results obtained in these more complex systems may be lower. The level of 78NP in plaque could be determined, including in which areas and stages of plaque 78NP is present.

78NP may protect other cell types in the lesion, such as smooth muscle or endothelial cells. These cell types may require a lower concentration of 78NP for an antioxidant effect, since they do not produce 78NP themselves. 78NP would be likely to have a greater stabilising effect on plaque if it could prevent loss of the smooth muscle cell cap of the acellular lipid core.

While the focus of this thesis has been on examining the macrophage death which contributes to the formation of the core, 78NP might affect other stages of plaque development. A possible influence on the oxidation of LDL by monocytes and macrophages has already been explored (Gieseg and Cato, 2003).

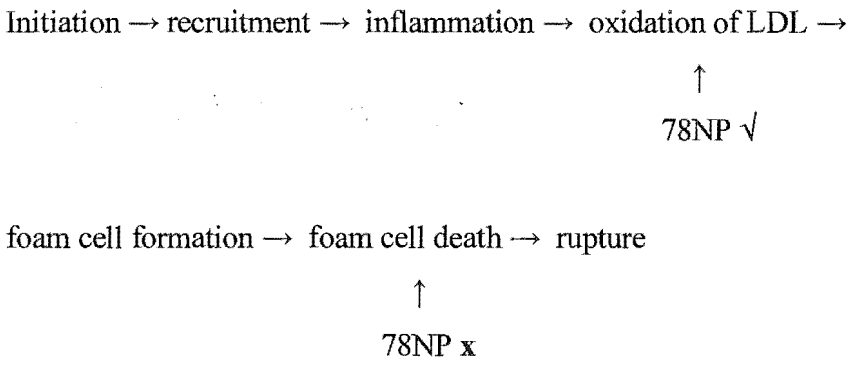

Figure 108: 78NP and the development of atherosclerosis.

Other ideas for functions for 78NP could also be tested. The fact that 78NP and neopterin are made from nucleotide bases may have some significance. Adenine can form a complex with two Cu^{2+} ions, which then has a catalase effect, disproportionating hydrogen peroxide to oxygen and water, while the complex itself slowly oxidises over around forty reactions (Bruston *et al.*, 1999). 78NP or neopterin may be able to interact with bases within DNA, perhaps protecting them from oxidative damage.

The possibility that 78NP might heighten macrophage immune response, suggested in the introduction, could also be tested. In this case, 78NP would encourage plaque and inflammation development.

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